Bacterial survival and decontamination in relation to food contact surfaces.

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BACTERIAL SURVIVAL AND DECONTAMINATION IN RELATION TO FOOD AND FOOD CONTACT SURFACES.

by

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"Messieurs, c'est les microbes qui auront le dernier mot"

("Gentlemen, it is the microbes that shall have the last word")

Louis Pasteur

ABSTRACT.

BACTERIAL SURVIVAL AND DECONTAMINATION IN RELATION TO FOOD AND FOOD CONTACT SURFACES

Claire Cassar

Foodborne illness is increasing worldwide and reported cases in Europe have tripled in the past ten years. Microbial contamination of foodstuffs may occur *via* food contact surfaces used in their preparation and processing. In addition, the surfaces of animal carcasses may be faecally contaminated prior to slaughter. This thesis is concerned with (i) the influence of the type of food contact surface on bacterial survival and ease of decontamination and (ii) the development of novel methods for killing bacteria on food surfaces, particularly chicken skin.

The survival of bacteria on impervious food contact surfaces (ceramic, glass, plastics, stainless steel) was investigated using an *in situ* technique. Samples of test surfaces were overlaid with agar and colonies formed after incubation visualised by reaction with nitroblue tetrazolium. The method was approximately sixfold more efficient in detecting organisms than a conventional swabbing technique.

It was shown, using the *in situ* method, that survival on surfaces was influenced by several parameters. Bacteria survived well in liquid films but on drying there was generally a large reduction in viability. The suspending medium in which organisms were applied to surfaces was a major factor influencing survival. Survival was poor for

cells suspended in deionised water (e.g. 0.0004 % for *Escherichia coli* and 0.4 % for *Staphylococcus aureus*). However, survival was greatly increased when cells were suspended in NaCl (0.1 to 10 % w/v) sucrose (0.1 to 10 % w/v) or serum (0.1 to 10 % w/v). In serum, survival rates of up to 0.01 % and 100 % were found for *E. coli* and *Staph. aureus*, respectively.

In all suspending media, the survival rate of Gram-positive organisms (*Staph. aureus* and *Listeria monocytogenes*) was consistently greater than that of Gram-negative organisms (*E. coli, Salmonella enteritidis* and *Pseudomonas fluorescens*). Physical factors (temperature and relative humidity) and surface type were less important than the nature of the suspending medium. Surface type also had little effect on the ease of removal of bacteria by washing with water or detergents. More than 90% of bacteria dried in serum or sodium chloride solutions were removed by a two minute water wash and the effectiveness of washing procedures was enhanced by both industrial and household detergents.

In contrast, bacteria dried on the surface of chicken skin were not readily removed by washing. Thus, novel decontamination procedures were investigated involving treatments based on exposure to trisodium phosphate (TSP) in combination with cold shock, osmotic shock and lysozyme. Trisodium phosphate has been shown to kill Gram-negative bacteria on the surfaces of chicken carcasses; however only high concentrations (approximately 10% w/v) are effective. The most successful combined procedure was to expose cells firstly to TSP (< 10mM) in the presence of 0.8M sodium chloride and then to lysozyme in deionised water. High kills (up to 99.99%) were obtained for suspended cells of *E. coli, S. enteritidis, P. fluorescens* and *Campylobacter jejuni*. The killing of attached organisms on chicken skin was less efficient but kills were still sufficiently high (approximately 95%) to suggest the feasibility of commercial applications.

PUBLICATIONS.

1. Miles, R. J., **Cassar, C.**, and Carneiro de Melo, A. (1995). Bacterial decontamination of foods. Patent application: PCT/GB 96/03173. Publication date 3 July 1997.

2. Barnes, B. I., **Cassar, C. A.**, Halabalab, M. A., Parkinson, N. H. and Miles, R. J. (1996). An *in situ* method for determining bacterial survival on food preparation surfaces using a redox dye. *Letters in Applied Microbiology* **23**, 325-328.

3. Carneiro de Melo, A., **Cassar, C.** and Miles, R. J. (1998). Trisodium phosphate increases sensitivity of Gram-negative bacteria to lysozyme and nisin. *Journal of Food Protection.* In press.

4. **Cassar, C.**, de Melo, A., Chatzopoulou, A. and Miles, R. Decontamination of poultry using lysozyme or nisin. Abstracts of the 3rd Hygienic Food Processing Workshop of the MAFF/ BBSRC LINK Advanced Food Manufacturing Programme, London, 22nd November 1996.

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DEDICATION.

I dedicate this thesis to my father and mother, Frank and Dorothy Cassar.

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ABBREVIATIONS

APC	Aerobic Plate Count
BHI	Brain Heart Infusion
DT	Definitive phage type
DVLO	Derjaguin-Landau-Verwey-Overbeck theory
EDTA	Ethylenediaminetetra-aceticacid
E_h	Redox Potential
FDA	Food and Drug Administration
GRAS	Generally Regarded as Safe
HACCP	Hazard Analysis Critical Control Point
NA	Nutrient agar
NAG	N-acetyl glucosamine
NAM	N-acetylmuramic acid
NBT	2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride
NCIMB	National Collection of Industrial and Marine Bacteria
NCTC	National Collection of Type Cultures
OD	Optical Density
PT	Phage type
RH	Relative Humidity
SEM	Scanning electron microscopy
SRM	Swab recovery medium

TSP	Trisodium phosphate
USDA	United States Department of Agriculture
WHO	World Health Organisation

SECTION I.

General Introduction

1.1. Microbial attachment to surfaces and the formation of biofilms.

Microorganisms preferentially attach to surfaces because nutrients are more concentrated at interfaces and are therefore more readily available for metabolism (Fletcher, 1991). Growth of attached organisms will eventually lead to microcolony formation and when the aggregation of cells becomes sufficiently large, it will trap debris, nutrients and other microorganisms and a microbial biofilm will be established (Fig.1.1). Microbial biofilms were first described by ZoBell (1943), who found that marine organisms present in seawater were able to attach to several types of immersed surfaces including glass, metal and plastics. Since then, microbial colonisation has been documented on such diverse surfaces as minerals within natural aquatic environments (Fletcher, 1987; Costerton *et al.*, 1978), the hulls of sea-going vessels (Lewin, 1984), medically implanted devices (Verheyen *et al.*, 1993), water distribution pipes (LeChavellier *et al.*, 1988) and industrial food contact surfaces (Bower *et al.*, 1996; Zottola and Sasahara, 1994).

In the food industry, the immobilisation of microorganisms may be advantageous, leading to increased productivity of bioreactors for fermented food production. In waste water treatment, microorganisms within biofilms also facilitate removal of both organic and inorganic compounds from waste water. However, in many situations, the attachment of microorganisms to surfaces and the formation of biofilms is clearly undesirable. This is particularly the case where food spoilage or pathogenic microorganisms adhere to food or food contact surfaces.

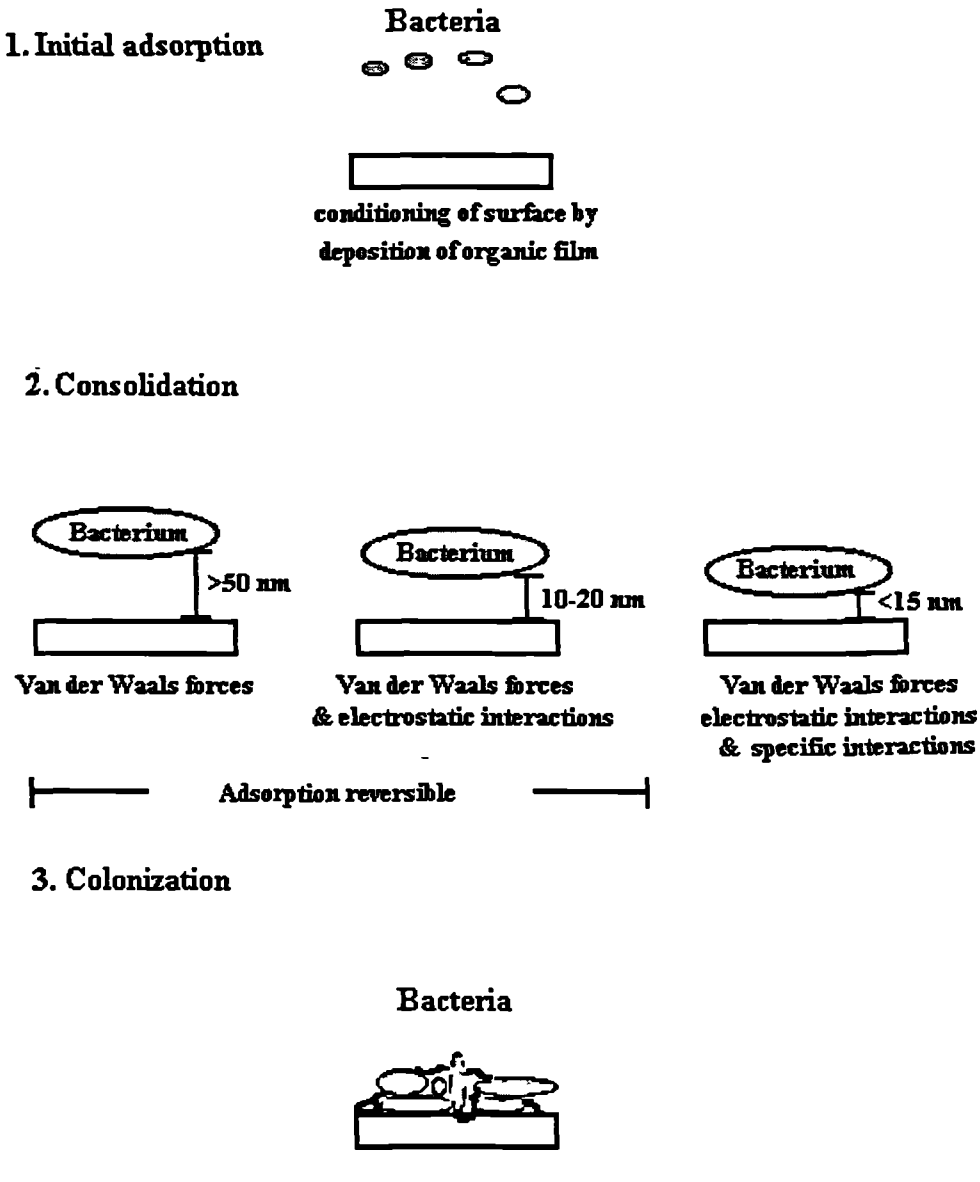


Fig. 1.1. Microbial adsorption to surfaces and the formation of biofilms. Adsorption is initially reversible. As colonisation develops, debris becomes trapped within the film. (Adapted from Zottola and Sasahara, 1995.)

1.2. The initial interactions of microorganisms with surfaces.

Biofilm formation is a complex process, comprising a number of distinct stages (Fig. 1.1). Marshall *et al.* (1971), proposed that attachment was a two-step process. The first stage was reversible and attributable to a combination of weak electrostatic attraction and Van Der Waals forces. The second stage involved physical bonding of microorganisms to the surface and was considered to be a time dependent and irreversible event, mediated by microbial production of complex polysaccharide (Costerton *et al.*, 1978). In an extension of the model of Marshall *et al.* (1971), Characklis and Cooksey (1983) emphasised the importance of the adsorption of organic and inorganic nutrients on the surface prior to microbial attachment. They also recognised that microbial metabolism and growth would eventually lead to detachment of the biofilm from the surface.

Busscher and Weerkamp (1987) recognised three steps in the process of attachment as organisms approach a surface (Fig.1.1). At distances greater than 20 nm, long-range forces such as electrostatic attraction and Van Der Waals forces are dominant and, at this stage, adhesion is a reversible process. As the distance between microbe and surface is reduced to 20 nm, both long-range forces and shorter range forces, such as hydrophobic interactions and chemical bonding, influence the adhesion process. At this stage, adhesion is initially reversible but may become irreversible with time. At distances of less than 15 nm, additional factors such as exopolysaccharide production, may also contribute to the attractive forces so promoting a firm adhesion. The models of Marshall *et al.* (1971) and Busscher and Weerkamp (1987), both assume that attached microorganisms are metabolically active and able to produce the adhesive polymers required for consolidation of biofilm development. The polymer produced is the basis of the glycocalyx which eventually binds and encloses the total microbial population.

The process of microbial surface adhesion has also been explained in terms of the ability to overcome an "interaction barrier". This "interaction barrier", first proposed as part of the Derjaguin-Landau-Verwey-Overbeck (DLVO) theory, may be considered as a "high

energy repulsion barrier" which is strongly influenced by the surface area of a particle (van Loosedrecht *et al.*, 1989, 1990). Once the "interaction barrier" has been overcome, microorganisms are able to establish microcolonies with subsequent biofilm formation. Microbial structures potentially important in overcoming the barrier include flagella and pili, in addition to capsular polysaccharides.

1.3. Factors influencing microbial attachment to surfaces and biofilm formation.

1.3.1. Cell surface charge.

Surface charge and hydrophobicity are important factors in the initial interaction of microbial cells with a surface and determine the length of time that the microbe remains in close contact with the surface. The surfaces of both Gram-positive and Gram-negative bacteria have a net negative charge at neutral pH (Ward and Berkeley, 1980); however, charge is not evenly distributed over the microbial surface. In Gram-positive organisms, the major surface constituents contributing to the negative charge are teichoic and teichuronic acids of the cell wall and the acidic polypeptides and polysaccharides of the cell capsule. In Gram-negative bacteria, the major constituents in addition to capsular polymers, are acidic lipopolysaccharides and proteins of the outer cell membrane. The significance of the net negative charge of bacterial cells for the complex attachment process are not known (Hood and Zottola, 1995).

1.3.2. Cell surface hydrophobicity.

It has been proposed that a major factor promoting bacterial adhesion to surfaces, is the rejection of hydrophobic cells from aqueous phases and their attraction towards non-aqueous phases such as solid surfaces (Marshall and Criuckshank, 1973). Microbial cell surface hydrophobicity varies significantly amongst organisms (Marshall and Criuckshank, 1973; Rosenberg *et al.*, 1980) and is markedly affected by environmental conditions (Fletcher, 1977). In Gram-negative bacteria, the outer membrane of the cell

wall is primarily responsible for these hydrophobic properties. However, although hydrophobicity is considered to play an important role in nonspecific microbial adhesion (Fattom and Shilo, 1984; Hood and Zottola, 1995) and may contribute to the orientation of certain microorganisms at interfaces (Marshall and Criuckshank, 1973), it is unclear in the literature whether hydrophobicity is quantitatively correlated with the extent of adherence.

1.3.3. The presence of adsorbed nutrients at the interface.

Microbial attachment may be influenced by the presence of soluble material adsorbed to a surface. Generally, surfaces that are exposed to nutrients such as proteins and carbohydrates tend to become quickly coated by these nutrients; in consequence the interface between the bulk fluid and the surface becomes significantly altered. Adsorbed nutrients may be retained at the surface where they may influence microbial attachment and detachment; alternatively they may be displaced from the surface by other components of the bulk fluid with a higher affinity for the surface (Marshall, 1985). The nutrient adsorption process of surfaces is dependent on several unrelated factors, including the hydrophobicity and electrokinetic charge of the surface, the hydrophobicity of the nutrient and any conformational change that nutrients may undergo during adsorption (Norde, 1986).

In early work on microbial attachment to surfaces, it was speculated that exopolymers were responsible for adherence (Marshall *et al.*, 1971). Consequently, it was thought that nutrient limitation would decrease adherence due to decreased polymer production. However, Wrangstadh *et al.* (1986) observed that when a range of microorganisms were starved of nutrients, so decreasing polymer production, microbial adhesion to glass increased. The reasons for such observations remain unexplained but it has been speculated (Hood and Zottola, 1995) that microorganisms might be specifically adapted to adhere to surfaces, under conditions of low nutrient availability, since the concentration of available nutrients at the surface will be greater than in the surrounding medium.

Conversely, the presence of high nutrient concentrations may saturate cell surface receptors, which would therefore be unavailable to bind nutrients present at the surface (Brown *et al.*, 1977).

1.3.4. The nature of the substratum surface.

The composition of the substratum surface can affect the number of adhered microbes and their distribution either directly by the nature of the surface or indirectly via effects on bacterial activity. Bacterial adherence is influenced by the substratum surface free energy and maximal values of bacterial adherence are observed on highly charged surfaces such as glass or polystyrene (Dexter *et al.*, 1975). The properties of a surface will also influence the type of bacterial polymers involved in attachment (Paul and Jeffrey, 1985); cell surface lipids or non-polar groups attached to polysaccharides may play an active role in adhesion to hydrophobic surfaces, whereas on hydrophilic surfaces, bacterial adhesion may be initiated by hydrated polysaccharides. The distribution of hydrophobic and hydrophilic components on the surface of microorganisms may vary. The microscopic finish of manufactured surfaces may also influence microbial adhesion (Speers *et al.*, 1984; Stone and Zottola, 1985). Scanning electron microscopy has revealed that the surface finishes applied to stainless steel lead to differences in the frequency of fissures and surface irregularities. These may act as potential microbial reservoirs and reduce the efficacy of cleaning regimes.

Once adhesion has taken place, substratum surfaces may also affect bacterial activity by influencing physiological processes such as substrate transport and energy conservation; these influences are thought to arise as a consequence of the unique physiochemical conditions that exist at solid-liquid interfaces (Bright and Fletcher, 1983). However, although the nature of the substratum surface has a significant effect on microbial adhesion and colonisation, there are many other important factors in this process, including the nature and background of the organism and the concentration and the type(s) of substrate available (van Loosedrecht *et al.*, 1990).

1.3.5. Exopolymer production.

Once bacteria have attached to a surface, nutrient dependent microbial growth may follow. Microbial growth on surfaces is typically accompanied by the production of extracellular polymers. These consist primarily of polysaccharides, most commonly with acidic properties (Herald and Zottola, 1988a; Zottola, 1991) and range from simple homopolymers of glucose or fructose to complex heteropolymers containing a diverse range of sugars. In electron microscope studies, polysaccharide material appears amorphous or as fibril-like structures, which stretch between the microbes and the substratum surface. Alternatively, the polysaccharide may form hydrated gel-like matrices within which cells are organised in complex multilayers (Lawrence *et al.*, 1991).

In addition to polysaccharides, other polymers such as proteins may also have a significant role in adhesion. Interestingly, proteases are more efficient than carbohydrate-hydrolysing enzymes in detaching microbes from surfaces. Thus, it has been suggested that surface proteins may have a role in the stabilisation of cell-substratum adhesion interactions (Fletcher and Marshall, 1982; Paul and Jeffrey, 1985; McEldowney and Fletcher, 1986).

In addition to effects on adhesion, the production and accumulation of exopolymers significantly influences the characteristics of the microenvironment at the substratum surface (Geesey, 1982). As the thickness of the biofilm increases, cells will become immobilised and the hydrated polymer matrix will present a substantial barrier to diffusion of nutrients, oxygen and toxic metabolic products. However, the presence of polymer matrices may protect adhered cells from external physical and chemical influences; it will, for example, modify redox potential (E_h) values and reduce the efficacy of disinfection procedures (Geesey, 1982). The matrix will also stabilise extracellular enzyme activity (Burchard, 1981). In addition, it is the presence of the polymer matrix which ultimately enables the populations of biofilms to reach high levels. These populations may contain

diverse microorganisms amongst which there may be competitive or synergistic interactions.

1.4. Microbial adherence and biofilm formation on the surface of meat and poultry.

The microbial contamination of animal carcasses during slaughtering procedures is undesirable but cannot be avoided. Microorganisms are effectively absent from the muscle tissue of live healthy animals; primary contamination of carcass surfaces commonly arises as a result of contact with dust, dirt and faecal material accumulated on hides, hooves, hair or feathers during the slaughtering process. Rumen fluid or intestinal contents can also act as initial sources of contamination.

There has been extensive research on the microbial populations attached to the surfaces of meat and poultry carcasses (Selgas *et al.*, 1993). One aspect of this research has focused on the diversity and size of populations. The results of such research are important for assessing public health and safety, estimating the efficacy of sanitary handling during processing and evaluating the sustained quality of the processed product. Other research has concentrated on the mechanisms and factors which may contribute to attachment. Some of these factors, such as cell surface charge, hydrophobicity and extracellular polysaccharides have been discussed in general terms in the preceding sections (Sections 1.3.1., 1.3.2. and 1.3.5). Factors considered important in attachment to the surface of meat and poultry carcasses are considered in the following sections (Sections 1.4.1., 1.4.2. and 1.4.3) together with the types of organisms commonly found on such surfaces.

1.4.1. The nature of meat and poultry surfaces.

Meat is a structurally and chemically heterogeneous substrate. It comprises lean, fat and connective tissues in variable proportions dependent on many factors including the sites within the animal, the particular breed of animal and the feeding regime. The chemical

composition of poultry and red meats is essentially similar, though poultry muscle has less fat and typically, a higher pH value.

Bacterial attachment to meat and poultry is complex and not fully understood. When bacteria first become associated with animal tissues, they may be present as either unattached cells within the water film at the tissue surface or be physically attached to the surface (Firstenberg-Eden *et al.*, 1978). Microbial attachment is enhanced by a number of factors intrinsic to the nature of the meat and is generally regarded as difficult to reverse. The distribution of surface charge on lean muscle and fat cells differs and will influence the kinetics of bacterial attachment. The kinetics of microbial attachment to meat will also be influenced by the nature of the microbial species and particularly, its cell surface charge (Dickson and Koohmaraie, 1989). Benedict *et al.* (1991) found it more difficult to reverse the attachment of *Salmonella* spp. to whole skin than to muscle-free skin and concluded that both surface charge and interacting surfaces contributed to the strength of attachment.

Despite the evidence for the role of electrostatic interactions in attachment, the involvement of microbial cell adhesins and specific attachment sites on the meat surface has also been considered. Piette and Idziak (1992) specifically attributed bacterial adherence to attachment sites on the collagen and proteoglycans of meat tendon. Schwach and Zottola (1982) had previously reported that *Pseudomonas fragi* appeared to be entrapped within collagen fibres of raw beef and Thomas and McMeekin (1981a) showed that *Salmonella* spp. similarly attached to collagen fibres of chicken muscle but only when the muscle sample had been previously immersed in water or saline for a prolonged period.

The study of Thomas and McMeekin (1981a) also demonstrated the role of surface microtopography in bacterial entrapment, since immersion of chicken muscle in water or physiological saline caused the collagen component of connective tissue to expand and form a dense mass of interconnecting fibres on the surface. Thomas and McMeekin

(1982) also reported time and temperature dependent changes in the microtopography of poultry skin during water immersion processing procedures. Water absorption by poultry skin caused capillary-size channels and spaces to open up in the surface layers of the skin. These would permit bacteria present in processing water to penetrate deep into the surface of the skin from where removal would be difficult. Thus, Kim *et al.* (1996) suggested that water entrapped in chicken skin feather follicles and crevices, during the immersion chilling stage of poultry processing, might act as a reservoir for *Salmonella*.. Other studies (Thomas and McMeekin, 1984; Lillard, 1986) have also concluded that water uptake accounts for the majority of bacterial contamination on the surface of chicken skin.

Bacterial attachment to the fat tissue and lean muscle of red meats has been compared. Dickson (1988) demonstrated that when beef muscle and fat tissues were washed with a selection of antimicrobial compounds, significantly more organisms were removed from the fat tissue. This difference was attributed to both the presence of protective factors on muscle tissue and increased physical removal of organisms from fat tissues due to saponification of the upper fat layers. There appears to be no significant difference between the numbers of organisms which initially attach to fat tissue and muscle tissue (Chung *et al.*, 1989; Dickson and Macniel, 1991).

1.4.2. Bacterial structures associated with attachment to meat and poultry.

Several studies have investigated the role of bacterial surface structures in attachment to meat and poultry. Notermans and Kampelmacher (1974) and Schwach and Zottola (1982) concluded that extracellular structures such as flagella could be implicated in microbial attachment to meat surfaces, and Butler *et al.* (1979) observed that adherence of motile Gram-negative organisms was greater than that of non-motile Gram-positive species. The ability of *Pseudomonas fluorescens* to adhere to tendon was promoted by the presence of flagella (Piette and Idziak, 1991). However, their role may be indirect, influencing adhesion by increasing the number of cells reaching the surface within a

specified time. Flagella were not essential for adhesion since deflagellated cells were also capable of adhering to the meat surface in high numbers.

In studies using chicken skin, there is little difference between the attachment of flagellated and non-flagellated organisms (Meadows, 1971; Lillard, 1985) and the ability to attach to the skin surface was independent of motility (Notermans *et al.*, 1980). Lillard (1985) concluded that flagella were not directly involved in attachment mechanisms, but may enable chemotaxis towards nutrients on the chicken skin surface.

1.4.3. Additional factors influencing bacterial attachment to meat and poultry.

Other factors which may affect the attachment of microrganisms to meat and poultry surfaces are pH, temperature and cell background. The attachment of *Salmonella typhimurium* and *Listeria monocytogenes* to beef tissue (Dickson, 1991) was greater on fat tissue at 23 °C than at higher temperatures. However, temperature (23 to 37 °C) had no influence on the attachment of either bacterial species to beef muscle. Notermans and Kampelmacher (1974) found that pH affected the rate of bacterial attachment to chicken skin, but the effect was species dependent. Dickson (1991) showed that increasing experimental inoculum levels resulted in corresponding increases in bacterial attachment to beef. However, early stationary phase cells of *Salmonella typhimurium* and *Listeria monocytogenes* attached in greater numbers than cells of stationary phase cultures which had been incubated for 66 h (Dickson, 1991). Similarly, microorganisms subjected to starvation stresses generally attached to the surface of beef in lower numbers than control organisms harvested in the early stationary phase (Dickson and Frank, 1993). There are many structural, physiological and metabolic differences between growing and non-growing cells, and cells grown at different growth rates (Pirt, 1976). Factors important in influencing attachment may include cell size, the amount of capsular material and the availability of endogenous energy reserves which will affect surface charge and motility.

1.5. Biofilms in food processing and on food contact surfaces.

Microbial contamination is the most important of the factors which may compromise food product quality and safety. Zoltai *et al.* (1981), were the among the first to report the attachment of bacteria to food contact surfaces. They showed, by scanning electron microscopy (SEM), the attachment of *Pseudomonas fragi* and *Staphylococcus aureus* cells to stainless steel and glass surfaces. They also demonstrated that increasing the contact time with the bacterial suspension, resulted in an increase in the number of attached cells. Herald and Zottola (1988a) similarly utilised SEM to demonstrate the presence of attachment fibrils in the adhesion process of *P. fragi* to stainless steel.

Subsequent investigations of microbial attachment and growth have mainly involved model systems. A variety of inert food contact surfaces have been used, often preconditioned with organic material. The factors investigated have included pH, temperature, relative humidity (RH), cell suspension medium and organism and surface type. The results obtained are described in the following sections which consider adherence and growth or survival on: food contact surfaces except wood (Section 1.5.1); wood (Section 1.5.2); and non-food contact surfaces (Section 1.5.3).

1.5.1. Factors affecting attachment and biofilm formation on food contact surfaces.

There have been a small number of studies using pure cultures of microorganisms, inoculated onto test surfaces in media of various composition and pH, and incubated under differing conditions of temperature and relative humidity.

Herald and Zottola (1988a) demonstrated the attachment of *L. monocytogenes* to stainless steel at a range of pH (5-8) and temperature (10 to 35 °C) values within the normal growth range of the organism. Mafu *et al.* (1990) reported the attachment of *L. monocytogenes* to stainless steel, polypropylene, rubber and glass surfaces after contact times of 20 minutes at both ambient (20 °C) and refrigerated temperatures (4 °C). Suárez *et al.* (1992)

investigated the adherence of psychrotrophic bacteria isolated from raw milk to stainless steel, rubber and glass surfaces. The levels of adherence of Gram-negative bacteria were higher than those of Gram-positive bacteria, presumably due to the effects of differences in their cell wall structure on such factors as hydrophobicity and cell surface charge. Chamberlain and Johal (1987) reported similar results for cells suspended in pork liquor.

Several workers have investigated the effect on surface attachment and survival of a given bacterial species, resulting from the presence of other species. Sasahara and Zottola (1993) investigated biofilm formation of *P. fragi* and *L. monocytogenes* on glass and stainless steel. They observed that attachment of *L. monocytogenes* only took place when grown in the presence of a recognised exopolysaccharide-producing microorganism like *P. fragi.* Banks and Bryers (1991) investigated bacterial species dominance within a binary biofilm comprising *Pseudomonas putida* and a *Hyphomicrobium* sp. The faster growing *P. putida* became rapidly established as the dominant species. However, the slower growing *Hyphomicrobium* sp. remained established within the biofilm and increased in number over time. Wirtanen and Mattila-Sandholm (1993) investigated the growth of a biofilm comprising *Bacillus subtilis, L. monocytogenes, Pediococcus pentosaceus* and *P. fragi* on stainless steel. After a growth period of 10 days, the majority of the organisms detected by epifluorescence microscopy, were not culturable.

Studies involving food contact surfaces preconditioned with organic material have been widely used within the food industry. The conditioning films most commonly used consist of proteins from milk or meat. Extensive research has been carried out to determine their effect on bacterial adhesion to such surfaces as stainless steel, buna-N rubber, glass and silica. Speers *et al.* (1984) initially demonstrated the proliferation of *Pseudomonas* spp. in whole milk adsorbed to glass and stainless steel but not rubber. However, Speers and Gilmour (1985), subsequently demonstrated that although preconditioning dairy equipment surfaces with non-casein proteins or lactose gave rise to high levels of microbial attachment, no increase in microbial attachment was observed in the presence of whole milk. Al-Makhlafi *et al.* (1994, 1995) investigated the effects of

preadsorbed milk proteins, singly and in combination, on the adhesion of *L. monocytogenes* to hydrophobic and hydrophilic silica surfaces. Adhesion was increased by the presence of protein but the extent of adhesion was influenced by protein type. The lowest levels of adhesion were obtained using milk albumin. Results were similar for both hydrophobic and hydrophilic glass surfaces. In contrast, Helke *et al.* (1993) found that conditioning stainless steel and buna-N rubber, with milk casein and β-lactoglobulin, actually decreased the adherence of *L. monocytogenes* and *S. typhimurium.* However, in agreement with previous observations (Speers and Gilmour, 1985), lactose was observed to have a small positive influence on adhesion of both microorganisms.

Helke and Wong (1994) showed that the attachment and growth of *L. monocytogenes* and *S. typhimurium* to stainless steel and buna-N rubber was influenced by temperature (6 to 25 °C), relative humidity (RH), type of soil and surface. On both surfaces, the survival of organisms grown at 6 °C, was higher at 75% RH than at 30% RH, partly due to a decreased rate of moisture loss enhancing survival of bacteria adhered to the surface. Similarly, Humphrey *et al.* (1995) observed that when *Campylobacter jejuni* suspended in blood was inoculated onto formica surfaces, viability was sustained whilst held in a liquid environment, but the organism was very sensitive to drying.

1.5.2. Microbial adhesion and biofilm formation on wooden surfaces.

Borneff *et al.* (1988) established that working surfaces including wood and plastic boards acted as one of the main sources of contamination in a household kitchen. Gilbert and Watson (1971) originally asserted that viable bacteria recoverable from new and abraded "in use" wooden chopping boards, both before and after cleaning, were consistently greater than those recovered from formica, polyethylene or fibre laminate boards treated in the same manner. However, there has recently been renewed controversy in America regarding the use of wood as a commercial food preparation surface. Reports by Ak *et al.* (1994a, 1994b) challenged the long-held, largely anecdotal assertion that plastic cutting boards are more sanitary than wooden boards (Felix, 1993, Raloff, 1993). They reported

that 10 min after inoculating new wooden cutting boards with foodborne pathogens *(E. coli, Listeria innocua, L. monocytogenes* and *S. typhimurium*), viable bacteria could not be recovered. Conversely, bacteria adhered to new plastic cutting boards could be recovered up to 12 h after inoculation and microbial multiplication took place if the plastic boards were held overnight. These observations were essentially confirmed by Abrishami *et al.* (1994), although they recovered 10 % of the original inoculum of *E. coli* from new and used wooden cutting boards after rinsing. SEM analysis of wooden board samples revealed that bacteria were present within the structural xylem fibres and vegetative elements of the wood (Abrishami *et al.*, 1994). Subsequent work by Galluzzo and Cliver (1996) further confirmed these results. They found that *S. enteritidis* rapidly became unrecoverable from oak cutting boards. It was suggested that the failure to recover inoculated bacteria from wood may have been due to physical factors, i.e. greater adhesion to the wood surface or the effects of wood on the drying of bacterial suspensions. Consequently the use of wooden cutting boards may be safer than previously believed. However, a recent report by Miller *et al.* (1996) claimed to find statistically similar patterns of attachment, survival and ease of removal of mixed bacterial populations from beef on polyethylene and wooden cutting boards. A subsequent report by Park and Cliver (1996) recommends the disinfection of wooden chopping boards by a brief exposure to microwaves in a conventional household microwave oven. This procedure was shown to efficiently disinfect wooden boards, but a similar treatment of artificially contaminated plastic boards did not significantly reduce the attached bacterial population. Although there appears to be conflicting reports in the literature concerning the survival of bacteria on wooden surfaces, the experimental approaches in the studies have varied. The variables have included the type of synthetic polymer used in the manufacture of plastic boards, the species of tree used for preparation of wooden boards, the bacterial inoculation method used (i.e. liquid cultures or naturally contaminated meats), prior use of the board (amount of wear), cleaning methods of boards and the method of bacteriological sampling and analysis.

1.5.3. Microbial adhesion and biofilm formation on non-food contact surfaces.

Several studies have been carried out to assess biofilm formation on non-food contact surfaces found in food handling, processing and catering environments. Non-food contact surfaces such as floor drains, wall tiles, and polyester floor sealant may act as reservoirs for foodborne pathogens. In food-processing plants, the accumulation of pathogenic microorganisms on such surfaces can lead to their spread throughout the factory (Pontefract, 1991). Nelson (1990) demonstrated the presence of *L. monocytogenes* in cast iron floor drains and Spurlock and Zottola (1991) showed low-level survival of *L. monocytogenes* in floor drains over a 28 day period. They concluded that, as the numbers of attached bacteria were low, the cells were probably loosely attached to the cast iron surface and could therefore become aerosolised during the cleaning of blocked drains. Blackman and Frank (1996) investigated the ability of *L. monocytogenes* to form a biofilm on a selection of surfaces used in food-processing plants, including stainless steel, nylon, Teflon and polyester floor sealant. Biofilm formation was observed on all of the surfaces following inoculation with cultures grown in trypticase soy broth. After 7 days, the highest population was recovered from the polyester floor sealant surface, the lowest was for nylon. It was concluded that the growth of *L. monocytogenes* in biofilms could potentially contaminate the food processing environment, if wet surfaces were not sanitised regularly.

1.6. Implications of microbial attachment and biofilm formation on food and food contact surfaces.

The ability of microorganisms to adhere to food and food contact surfaces, possibly leading to biofilm formation, is of great importance in both domestic and industrial environments (Zottola and Sasahara, 1994). In industry, it may lead to contamination of the final product which may in turn, result in a reduction of microbiological safety and quality. Generally, the numbers and types of organisms present in a food product are influenced by several factors, the most significant of which are: (i) the general

environment from which the food was originally obtained; (ii) the microbiological quality of the food in its raw or unprocessed state; (iii) the hygienic conditions under which the product is handled and processed; and (iv) the adequacy of subsequent packaging, handling and storage conditions in maintaining low levels of microorganisms. The failure to produce a 'safe' food product may have serious implications in the transmission of foodborne disease and spoilage of the food product. In the home, a major concern is the transmission of foodborne pathogens by cross contamination of foods, either directly or indirectly via food contact surfaces, particularly chopping boards.

1.6.1. Microorganisms involved in the spoilage of meat and poultry.

Meat and poultry are among the most perishable of all important food types. A number of interrelated factors influence their shelf-life and holding quality, specifically the holding temperature, atmospheric temperature, indigenous enzymes, moisture content, light and most significantly, their microbial populations. Each one of these factors alone or in combination can influence the storage time of the meat product. Despite the fact that some deterioration will occur in the absence of microorganisms, microbial activity is undoubtedly the most important factor in the deterioration of meat. Gill (1986) has defined meat spoilage as any single symptom or group of symptoms of overt microbial activity which is characterised by changes in odour, flavour or appearance.

A diverse range of bacteria have been isolated from fresh meats and poultry. The most frequently found are listed in Table 1.1. Since fresh meat is generally preserved at refrigeration temperatures the most prevalent organisms are psychrotrophs. Gram-negative psychrotrophs are recognised as the primary cause of spoilage, particularly *Pseudomonas*, *Moraxella* and *Acinetobacter* species (Gill, 1983) and the facultative anaerobe *Alteromonas putrefaciens* (Buchanan and Palumbo, 1985), which has now been reclassified as *Shewanella* (Jay, 1996). *Pseudomonas* species usually account for the majority of the bacterial population present on chilled meats stored under unmodified atmospheric conditions. The psychrotrophic pseudomonads, including *P. fluorescens*,

have a growth rate advantage over the majority of competing psychrotrophs and mesophiles at temperatures up to approximately 20 °C. However, as temperatures approach 30 °C, pseudomonads are replaced by mesophilic strains of *Acinetobacter* and *Enterobacteriaceae* such as *S. typhimurium* and *E. coli* (Gill and Newton, 1980a). The flora of poultry carcasses is also comprised largely of Gram-negative psychrotrophs, the most prevalent species being those of *Pseudomonas* and other closely related Gram-negative genera.

Microbial spoilage of red meats is a complex process which is generally confined to the surface of the product. Similarly, in whole poultry carcasses, spoilage takes place primarily on the skin (May, 1961). Meat and poultry contain high quantities of a diverse range of nutrients for bacterial growth and are therefore particularly susceptible to microbial spoilage. Initially, spoilage organisms are most likely to utilise the glucose component of surface tissue; once this has been depleted, lactic acid and other carbohydrates will be utilised. Some *Pseudomonas* species metabolise glucose catabolites such as gluconic and 2-oxogluconic acids (Farber and Idziak, 1982). Microbial protease production will also lead to the degradation of connective, sarcoplasmic and myofibrillar tissue enabling the subsequent catabolism of the free amino acids formed. The metabolism of organic and amino acids will lead to increased pH values. At this stage in the spoilage process, meat has a characteristically dark hue and is firm and dry to touch (Gill and Newton, 1980b). Microbial metabolism, particularly by Gram -ve bacterial species (Dainty, 1985) will also lead to the formation of foul odours. The catabolic products responsible for these 'off-odours' include: saturated and unsaturated aliphatic hydrocarbons; aromatic hydrocarbons; ketones, aldehydes, acids and alcohols; methyl and ethyl esters of short chain fatty acids; sulphur containing compounds such as sulphides and disulphides; and amines. Many of these products arise from amino acid catabolism.

In the final stages of meat spoilage the surface becomes 'tacky' and covered in 'slime'. This is mainly attributable to the production of exopolysaccharides by the increased microbial population present on the surface of the meat, but is also a consequence of the softening of meat tissues by proteolytic and other degradative enzyme activities.

Table 1.1. The genera of bacteria most frequently isolated from fresh meats and poultry (adapted from Jay, 1996).

Genus	Gram Reaction	Fresh Meats	Poultry
Acinetobacter	-	++	++
Aeromonas	-	++	+
Alcaligenes	-	+	+
Bacillus	+	+	+
Brochothrix	+	+	0
Campylobacter	-	0	++
Carnobacterium	+	+	0
Citrobacter	-	+	+
Clostridium	+	+	+
Corynebacterium	+	+	++
Enterobacter	-	+	+
Enterococcus	+	++	+
Escherichia	-	+	+
Flavobacterium	-	+	++
Hafnia	-	+	0
Kurthia	+	+	0
Lactococcus	+	+	0
Lactobacillus	+	+	0
Leuconostoc	+	+	0
Listeria	+	+	++
Microbacterium	+	+	+
Micrococcus	+	+	+
Moraxella	-	++	+
Pantoea	-	+	+
Pediococcus	+	+	0
Proteus	-	+	+
Pseudomonas	-	++	++
Salmonella	-	+	+
Serratia	-	+	+
Shewanella	-	+	0
Staphylococcus	+	+	+
Vagococcus	+	0	++
Yersinia	-	+	0

(++) frequently isolated ; (+) less frequently isolated; (0) rarely isolated.

1.6.2. Microbial foodborne disease.

1.6.2.1. Incidence and economic cost of foodborne disease.

The total incidence of foodborne disease in the United States is estimated at between 6 and 80 million illnesses per annum (Todd, 1991). In Europe, the number of reported food poisoning cases is approximately 50,000 per year in the UK (Sockett *et al.*, 1993) and 90,000 cases per year in Germany (WHO, 1992). However, the World Health Organisation speculate that only about 10% of incidents that take place in Europe are actually reported (WHO, 1992). The reported cases are most likely to be those where physicians have been consulted and where illness was more serious, or involved high risk groups, i.e. infants, geriatrics and the immunocompromised. Cases that are unreported are likely to be those where illness was mild and those affected were able to treat themselves.

Although many infectious diseases are declining in the developed world, the incidence of reported foodborne disease is increasing. In England and Wales, formal notifications of food poisoning cases increased fourfold during the 10 year period to 1995 (CDSC, 1996). Similarly, in Germany there was a doubling of reported cases between 1985-1989 (WHO 1992). It is possible that part of this increase is due to better reporting and improved diagnostic procedures. Nevertheless, it is generally believed that foodborne disease is increasing worldwide (Sockett, 1993).

Foodborne disease varies in its seriousness according to the organism involved. However, even in mild cases there is likely to be loss of working days. In the United States, the economic impact of foodborne disease has been estimated at more than 5 million dollars per annum (Altekruse and Swerdlow, 1996). Infectious intestinal disease, attributable to foodborne sources, is also responsible for approximately 9,000 deaths per year in the United States and 155 deaths per year in the UK (Djuretic *et al.*, 1996). Thus foodborne disease is of major economic and public health significance.

1.6.2.2. Organisms most frequently associated with foodborne disease.

A wide range of organisms have been shown to cause foodborne disease (Table 1.2; Baird-Parker, 1994). The importance of many of these, including *Campylobacter*, *Cryptosporidium*, enterohaemorrhagic *E. coli* and *Listeria*, has been recognised only since the 1950's. In terms of number of cases, *Campylobacter* and *Salmonella* species are the most significant of the food-borne organisms causing gastrointestinal disease (Figure 1.2), accounting for more than 75 % of the total cases in England and Wales (Stringer, 1994). Importantly, the number of cases caused by these organisms has risen consistently in England and Wales, during the period 1980 -1992 (Figure 1.2).

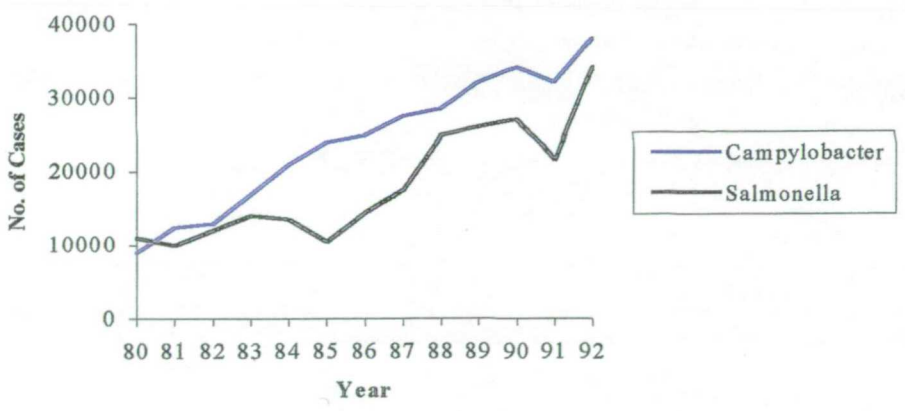


Fig. 1.2. Laboratory reports of gastrointestinal infections in England and Wales 1980-1992. (Adapted from Stringer, 1994.)

In terms of general outbreaks of foodborne disease (i.e. outbreaks affecting members of an institution or more than one private residence), the most important food poisoning organisms in the UK (Cowden *et al.*, 1995) are: *Bacillus cereus* (2 % of outbreaks), *Campylobacter* (2 %), *Clostridium perfringens* (15 %), *Escherichia coli* O157 (2 %), *Salmonella enteritidis* (29 %), *Salmonella typhimurium* (7 %), other *Salmonella* serotypes (4 %), and *Staphylococcus aureus* (2 %). Small round structured viruses (SRSV) also cause approximately 5% of outbreaks.

Food poisoning organisms have diverse origins, including human and animal faeces and environmental sources (Table 1.2). In addition, *Staph. aureus* may be transmitted to foodstuffs from the skin and nasal and oral secretions of food handlers. The major food poisoning organisms are considered below.

Table 1.2. Food poisoning organisms, classified according to origin.

Origin of food poisoning organisms		
Human	**Animal**	**Environment**
Enteroviruses*	*Bacillus anthracis*	*Aeromonas* spp.*
Escherichia coli	*Brucella* spp.	*Bacillus cereus*
enterotoxigenic		
enteropathogenic*	*Salmonella typhimurium*	
enteroinvasive*	*Salmonella enteritidis*	
Salmonella paratyphi B	*Campylobacter* spp.*	*Bacillus licheniformis**
Salmonella typhi	*Cryptosporidium* spp.	*Bacillus subtilis**
Shigella spp.	*Giardia* spp.	*Clostridium botulinum*
Staphylococcus aureus	*Listeria monocytogenes**	*Clostridium perfringens*
Streptococcus pyogenes	*Mycobacterium bovis*	*Vibrio parahaemolyticus*
		Other *Vibrio* spp.*
Vibrio cholerae	*Plesiomonas shigelloides**	
	*Streptococcus epidemicus**	
	*Toxoplasma gondii**	
	*Yersinia enterocolytica**	

* Organisms recognised as causing foodborne disease since the 1950's (Baird-Parker, 1994).

Salmonella is a major foodborne pathogen with a worldwide distribution. *Salmonella* may be transmitted *via* water used for drinking and food preparation. However, in the developed world, transmission appears almost exclusively to be foodborne. Again, in the developed world, *S. typhi* and *paratyphi* infections, associated with human faecal contamination, are rare; however, infections with other serotypes are widespread. These cause diarrhoeal disease, sometimes with fever and vomiting, following a short incubation period of 12 - 36 h. In the United States, food sources responsible for approximately 50 % of *Salmonella* disease outbreaks have been identified. The major food vehicles were beef, turkey, chicken, ice cream, pork, dairy products and eggs (Bean and Griffin, 1990). Similar foodstuffs have been identified as responsible for *Salmonella* transmission in the UK (Cowden *et al.*, 1995). In the UK, *S. typhimurium* was the most commonly isolated serotype up until 1988, but has since been superceded by *S. enteritidis* (58,000 cases in England and Wales during the period 1992-1994; Wall *et al.*, 1996). Amongst *S. enteritidis*, phage type (PT) 4 is predominant (48,000 cases in England and Wales 1992-1994) despite having fallen from a peak of 17,257 reported cases in 1993 to 12,351 in 1995 (Threlfall *et al.*, 1996). The number of *S. typhimurium* isolations, however, remain high (15,700 in the period 1992-1994; Wall *et al.*, 1996) and particularly for the phage type DT 104 (3,837 in 1995; Threlfall *et al.*, 1996). Interestingly, 97 % of these isolates exhibited resistance to one or more commonly used antibiotics, and most were resistant to at least four antibiotics (Glynn *et al.*, 1998). There were 19,000 isolations of other *Salmonella* serotypes in England and Wales between 1992 and 1994, of which almost 30% were *S. virchow* (Wall *et al.*, 1996).

Campylobacter is probably the most common cause of foodborne gastroenteritis in the developed world. In both Europe and the United States, *Campylobacter* infection is more common than that caused by *Salmonella* (Stringer, 1994; Altekruse and Swerdlow, 1996). Reported cases have risen dramatically from 12,168 in 1981 to 38,552 cases in 1992. The vast majority of cases occur sporadically and recognised outbreaks of disease (affecting more than one residence) are relatively few (466 in 1992). Consequently, the true incidence of *Campylobacter* enteritis is uncertain and may be considerably under-

estimated, especially as in the majority of cases the resulting illness is relatively mild and self limiting (Phillips, 1995). The most commonly reported food vehicle for the transmission of *Campylobacter* is milk, particularly unpasteurized milk. Poultry is also a common source of infection. It is estimated that between 30 and 100 % of retail broilers are contaminated with *Campylobacter* (Stringer, 1994). The most important *Campylobacter* species in relation to human foodborne disease are *C. jejuni*, *C. coli* and *C. fetus* subsp *fetus*. In animals, *Campylobacter* are frequently associated with abortion. In man they are associated with profuse and often bloody diarrhoea. *C. fetus* subsp *fetus* is particularly important in the immunocompromised, in whom it may cause septicaemia. Enteropathogenic, enteroinvasive, enterotoxigenic and enterohaemorrhagic strains of *E. coli* have all been associated with foodborne disease. However, currently the most important are the enterohaemorrhagic strains, the most widely recognised serotype of which is *E. coli* O157:H7. This has been the causative agent of foodborne disease outbreaks throughout the world, including such developed countries as the United States, Canada, Japan and the UK. There were 1,266 cases in England and Wales between 1992 and 1994 (Djuretic *et al.*, 1996) and the incidence of the disease is increasing (Thomas *et al.*, 1993).

Escherichia coli O157:H7 was first recognised as a pathogen in 1982, following its association with two food related outbreaks of unusual gastrointestinal illness (Wells *et al.*, 1983). It may cause particularly severe illness with haemorrhagic colitis, haemolytic uremic syndrome and thrombotic thrombocytopenic purpurea (Doyle, 1991). Both beef and dairy cattle, especially young animals within herds, have been recognised as reservoirs (Chapman *et al.*, 1989). Most *E. coli* O157:H7 outbreaks have been linked to the consumption of undercooked ground beef but several smaller outbreaks have been attributed to a variety of other foods including turkey roll (Ryan *et al.*, 1986), ham and turkey sandwiches (Carter *et al.*, 1987) and raw milk (Martin *et al.*, 1986).

Listeria monocytogenes is a relatively rare foodborne pathogen, but is associated with stillbirth, meningitis and septicaemia. In the United States successful collaboration between the food industry and government regulatory agencies reduced the incidence of listeriosis between 1989 and 1993 by 44 % (Tappero *et al.*, 1995). However, there are still almost 2,000 cases per year, with a mortality rate of approximately 25% (IFST, 1995). High risk groups are pregnant women, unborn or newborn infants, the elderly and the immunocompromised (Farber, 1993). An important attribute of the organism in relation to foodborne disease is its ability to grow at refrigeration temperatures. *Listeria monocytogenes* may be transmitted through a variety of contaminated foods particularly raw milk, meat, poultry and seafood.

In addition to the infectious foodborne organisms (some of which are described above), a number of bacteria are able to cause disease *via* toxins produced in the food prior to its consumption. Toxin production requires bacterial growth in the food and, at the time of consumption, viable cells of the food poisoning organism need not be present. The recognised food poisoning organisms which exert their effect *via* toxin production in foods are *Staph. aureus* and a number of spore forming bacteria. The latter include *B. cereus, B. licheniformis, B. subtilis, Cl. botulinum* and *Cl. perfringens.* The most common of the toxic food poisoning bacteria is *Staph. aureus. Staphylococcus aureus* food poisoning is ordinarily characterised by vomiting within 2 - 6 h after ingestion of contaminated food. Illness is not prolonged and data for total cases are not available. However, *Staph. aureus* is responsible for many major foodborne disease outbreaks. Typically, these arise from the consumption of inadequately refrigerated meat and dairy products, particularly cooked processed foods, which have been contaminated with *Staph. aureus* from the skin and nasal and oral secretions of food handlers (Bean and Griffin, 1990).

Foodborne disease attributed to *Clostridium perfringens* is relatively common. It accounts for approximately 15 % of currently reported foodborne disease outbreaks in England and Wales, though the frequency of outbreaks appears to be in decline (Djuretic *et al.*, 1996; Cowden *et al.*, 1996). Illness is characterised by diarrhoea and abdominal pain within 8 to 12 h of ingestion of contaminated food. *Clostridium perfringens* is a commensal intestinal organism of man and animals and is ubiquitous in the environment. Thus, food may become contaminated from a variety of sources. The ingestion of *Cl. botulinum* toxin in foods, or its production in the gut following the germination of ingested spores, frequently leads to death. Effects of the toxin become apparent within 12 to 36 h and lead to a progressive paralysis. The number of cases in the UK is very small and the source of contamination is environmental, *Cl. botulinum* being associated primarily with anaerobic muds and soils. *Bacillus* species, also found in soils, are becoming increasingly important as contaminants of cereal foods. The incubation period following ingestion of toxin is from 2 to 16 h, and the main symptoms are diarrhoea and/or vomiting. The most important of the *Bacillus* species is *B. cereus*.

1.6.2.3. Control of foodborne disease.

Statistics suggest that the incidence of foodborne disease in the developed world is increasing (Section 1.6.2.1). This trend including the emergence of 'new' foodborne pathogens reflects increased global travel and commerce, changing food industry demographics and technology and consumer demand for new and minimally preserved food products. Also, in the developed countries of the world, there is an increasing proportion of elderly people within the population, who have enhanced susceptibility to infectious disease. Contamination of foods by the common food poisoning organisms (Section 1.6.2.2) may arise from human, animal or environmental sources, but in many food products of animal origin, faecal contamination is particularly important. Faecal organisms in such foods may cause disease where foods have been undercooked. The introduction of faecal organisms into food preparation areas may also lead to direct or indirect cross-contamination of foods (De Wit *et al.*, 1979; Humphrey *et al.*, 1994). For

example, De Boer and Hahné (1990) showed that *C. jejuni* and *Salmonella* could be easily transferred from raw chicken products to cutting boards, plates and hands.

It may be unrealistic to suggest that all microbiological hazards pertaining to foodborne disease can be eliminated. The diligent implementation and maintenance of existing technologies such as pasteurisation, refrigeration and decontamination procedures in food processing plants are important in restricting the levels of foodborne disease incidence. However, there is a demand for novel technologies, particularly those that may enable the terminal decontamination of potentially heavily and/or faecally contaminated foods, such as chicken carcasses. Also, in food handling, much of the legislation and advice in relation to desirable practices is not based on detailed scientific evidence. Hazard Analysis Critical Control Point (HACCP) is an approach that can help identify points where control measures would be of benefit in relation to the processing, distribution and final preparation of foods. However, in many situations, such analysis may require more information than is currently available. For example, factors affecting the survival of microorganisms on surfaces, a major aspect of the experimental work reported here, is poorly understood. A detailed discussion of legislation and codes of practice relating to food processing and handling is outside the scope of this thesis.

1.7. Decontamination of food and food contact surfaces.

Many food surfaces have large, natural microbial populations. In addition, the contamination of food surfaces during food processing may be unavoidable. This is particularly so for meats and poultry, which are likely to be contaminated by faecal material. Decontamination of the surface of food products to significantly reduce such populations, would increase food safety and reduce spoilage. In addition, both during processing and the subsequent distribution, storage and preparation of foods, potentially pathogenic and spoilage organisms may gain access to foods from food contact surfaces. Therefore, a further requirement for reducing foodborne disease and increasing storage time of foods is effective sanitisation of food contact surfaces.

1.7.1. Decontamination of food contact surfaces.

In food processing plants, accumulations comprising both inorganic and organic matter (fats, proteins and carbohydrates) are referred to as 'soil' (Zottola and Sasahara, 1994). Cleaning programmes have been defined within the industry (Zottola and Sasahara, 1994) to inhibit the accumulation of soil and microorganisms on surfaces and thus prevent biofilm formation and contamination of foods. Detergents are used to penetrate and aid removal of soil and may include: alkalis, to emulsify fats and oils; dispersants, to solubilise proteins; chelating agents to bind and remove metal ions; acids, for removal of mineral deposits; deflocculating agents; and surfactants, to act as wetting agents. Following the application of detergent, sanitisers or disinfectants are applied to kill microorganisms remaining on the surface (Zottola and Sasahara, 1994). The most commonly utilised chemical disinfectants are quaternary ammonium compounds, hypochlorite or chloramines, iodophors, acids and biguanides (Sprenger, 1989). High temperatures or steam can also be used as forms of sanitising treatments. The selection of disinfectant is determined by several factors and may vary according to the application. Many sanitising agents, and particularly chlorine, are inactivated by organic compounds. Also, wetting agents are generally necessary to enable efficient contact of disinfectants with attached microorganisms.

The currently used disinfectants for food contact surfaces must be removed by thorough washing after completion of the cleansing procedure. This is necessary since they may be toxic and/or would taint food. However, Somers *et al.* (1994) have proposed the use of trisodium phosphate (TSP) as a possible disinfectant. Treatment with TSP has been approved for the pre-chill processing of chicken carcasses by the United States Department of Agriculture (USDA). Thus, it would appear suitable for use as a cleansing agent for food contact surfaces. Similarly, Oh and Marshall (1995) have proposed that acetic acid in combination with monolaurin, a GRAS approved food additive (Jay, 1996), might also be able to substitute for conventional disinfection procedures, and they showed that the combination was effective against surface-attached *L. monocytogenes*.

1.7.2. The efficacy of disinfectants in the decontamination of surfaces.

Suspension tests have been widely used to assess the activity of disinfectants under a variety of conditions. However, it is well documented that adhered microorganisms have greater resistance to bactericidal compounds than cells in suspension (Holah *et al.*, 1990; Mosteller and Bishop, 1993). Thus, in recent studies, the efficacy of disinfectants against microorganisms attached to surfaces and within biofilms, and physiological factors affecting the resistance of such organisms to disinfection, have been investigated.

Wirtanen and Mattila-Sandholm (1992a) investigated the efficacy of disinfectants against *P. fragi, B. subtilis, L. monocytogenes,* and *Enterococcus hirae* in suspension and also on steel surfaces where a biofilm had been allowed to develop. The results indicated that in practice, the suspension tests could not be used as indicators of microbial susceptibility in biofilms. Mustapha and Leiwen (1989) suggested that biofilm age influenced the resistance of *L. monocytogenes* to sodium hypochlorite and quaternary ammonium disinfectants. Similarly, Wirtanen and Mattila-Sandholm (1992b) showed that microbial resistance to chlorine increased with biofilm age. Furthermore, Frank and Koffi (1990) demonstrated that the removal of *L. monocytogenes* cells adhered to a surface increased their susceptibility to disinfectants to a level equivalent to that of cells in suspension. Bourion and Cerf (1996) examined biofilm formation by *L. innocua* and *P. aeruginosa* on stainless steel, teflon and rubber; they observed that *L. innocua* was much less sensitive to disinfectants (sodium hypochlorite/sodium thiosulphate and peracetic acid) in mixed biofilms where the cells appeared to benefit from the presence of *P. aeruginosa.* Cells within biofilms generally have lower growth rates (Evans *et al.*, 1991) and respiratory activity (Yu and McFeters, 1994) than suspended cells.

The increased resistance of microorganisms within biofilms to disinfectants is attributed to the slow diffusion of disinfectants through the biofilm. Microorganisms within biofilms have similarly been shown to receive less oxygen and fewer nutrients than cells in suspension (Brown *et al.*, 1988). In addition, Van de Weyer *et al.* (1993) showed that

the efficacy of nine disinfectants used in the food industry (QAC; cationic, anionic and non-ionic surface-active agents; aldehydes; alcohols; phenols; and chlorine compounds) against suspended cells of several *Listeria* strains, was reduced by the presence of organic matter.

One approach to overcoming the resistance to disinfectants, of microorganisms attached to surfaces, is to incorporate biocides within or at the surface of the material. This strategy has been used for sea-going vessels, which are coated with antibiofoulants such as tributyltin, to prevent biofilm formation (Bundy *et al.*, 1997). It has been proposed that such antibiofoulants may be of value in coating non-food contact surfaces within food processing plants (Eastwood, 1996), though the applications of compounds such as tributyl tin in the food industry will necessarily be restricted. Bower *et al.* (1995) have shown that silica surfaces may adsorb nisin at concentrations sufficient to kill surface attached cells of *L. monocytogenes*. Nisin is an effective inhibitor of many Gram-positive pathogens and food spoilage organisms (see Section 1.8.2.2.3). It is also an approved food additive (Owen Fields, 1996) and may therefore be suitable for use with food contact surfaces.

1.7.3. The effect of surface type on the efficacy of decontamination.

The cleaning of a food contact surface normally commences with the physical removal of soil. The effectiveness of this initial cleaning may be reduced by crevices and other surface imperfections which affect surface microtopography and therefore entrap or shield microorganisms. Krysinski *et al.* (1992) investigated the efficacy of a selection of cleaning and sanitising compounds on *L. monocytogenes* adhered to stainless steel or plastics. They concluded that the resistance of adhered cells to disinfectants or cleaners was dependent on the nature of the surface. Biofilms were more difficult to remove from plastics than stainless steel. Also, complete biofilm removal was only achieved when the surfaces were initially cleaned with detergent solutions. Mafu *et al.* (1990) similarly showed that porous surfaces such as rubber required five to tenfold greater concentrations

of disinfectants than those required for the removal of *L. monocytogenes* from stainless steel. Stevens and Holah (1993) investigated the effectiveness with which stainless steel, enamelled steel, mineral resin and polycarbonate surfaces could be decontaminated. When unused, results with all surfaces were comparable. However, after abrasive treatment, stainless steel was the most easily disinfected. Ak *et al.* (1994b) also demonstrated that, after inoculation with microorganisms and treatment with chicken fat, both new wood and plastic chopping boards were relatively easy to decontaminate. In contrast, used boards, and particularly plastic boards, were more difficult to clean.

1.7.4. Analytical methods for the determination of microbial survival on food contact surfaces.

A major problem in studies involving detection of microorganisms on surfaces, is the efficiency with which the attached microbial population may be sampled. There are a number of approaches. Classically, organisms are removed from the surface by swabbing or adhesion to agar and determined by viable counts. Swabs may be of cotton wool or alginate. The use of calcium alginate appears advantageous as alginate may be readily dissolved in aqueous media. However, recoveries of attached organisms from surfaces are lower using alginate than cotton wool swabs (Patterson, 1971). Using cotton wool swabs, it is desirable to swab first with a moistened swab and then a dry swab, and to pool the organisms so recovered (Humphrey *et al.*, 1995). The recovery solution used to moisten the swab, and resuspend organisms removed by swabbing, may contain Tween 80 (Sveum *et al.*, 1992), a surfactant which will also neutralise residual quantities of certain disinfectants used on surfaces (Sveum *et al.*, 1992). Recovery solutions also usually contain a buffer component and sodium chloride, to create an optimal water activity (osmotic strength). The solution used in this study (swab resuscitation medium; SRM) is described in Section 2.5.2. and was originally formulated by Dr. T. Donovan (cited by Humphrey *et al.*, 1995). However, there is evidence that the numbers of organisms recovered from surfaces by swabbing methods may be as low

as 10% (Musial and Rosenblatt, 1989) and that a significant factor may be the retention of some organisms by the cotton wool tip (Patterson, 1971; Holah *et al.*, 1988).

The most common agar contact methods use either an agar 'sausage' which may be rolled over the test surface or specially-prepared agar plates which have a raised agar surface (RODAC plates; Patterson, 1971). Recovery of *Bacillus subtilis* from stainless steel surfaces were similar for swab and agar contact (RODAC plate) methods (Angelotti *et al.*, 1964). Agar contact methods are only suitable for smooth flat surfaces and the maximum numbers of organisms which may be counted is only approximately 50 cfu cm^{-2} (Niskanen and Pohja, 1977). Thus, the method is unsuitable for heavily contaminated surfaces (Patterson, 1971).

In addition to viable counting techniques, microbial populations on surfaces have also been investigated by microscopy, particularly scanning electron microscopy (SEM; Zoltai *et al.*, 1981), and epifluorescence microscopy using acridine orange (Holah *et al.*, 1988). Microscopic techniques may be used for measuring low levels of surface contamination but may not be applicable in non-laboratory situations and, crucially, do not distinguish viable organisms (Holah *et al.*, 1988). A number of indirect techniques have also proved useful in analysing surface populations of microorganisms. These include analysis of proteins (Herald and Zottola, 1988) and exopolysaccharide (Wirtanen and Mattila-Sandholm, 1993) in surface films. However, the relationship of these to viable cell numbers is uncertain. Thus, there appears at present no unequivocal method for the accurate determination of viable cell numbers on surfaces.

1.8. Decontamination of fresh meat and poultry.

Extensive studies have been carried out in an attempt to develop methods which may be used to reduce the numbers of pathogens and spoilage organisms on fresh meat and poultry. A major barrier to the development of such methods is the effect of treatment processes on the characteristics of the raw product. Also, where treatments involve

exposure to chemicals, chemical residues may be toxic or alter the organoleptic qualities of the product. In addition, consumers are increasingly reluctant to purchase food regarded as heavily preserved or containing unnatural additives. Current and novel technologies for reducing bacterial numbers on fresh meat and poultry are listed in Table 1.3 and summarised in the following sections (Sections 1.8.1 and 1.8.2). The technologies are sub-divided according to whether they are based on physical or chemical means or are dependent upon the natural antimicrobial substances, lysozyme and nisin.

Table 1.3. Current and novel technologies for reducing bacterial numbers on fresh meat and poultry.

1. Procedures that inactivate foodborne microorganisms
Heat processing Irradiation High hydrostatic pressure (180 -700Mpa) Exposure to chlorine compounds Lysozyme treatment Bacteriocin treatment (principally using nisin) Lactoperoxidase systems Exposure to high concentrations of Trisodium phosphate (10 %w/v; pH >12) Exposure to organic acids (principally low molecular weight organic acids)
2. Procedures that inhibit growth of foodborne organisms
Chilled storage (0-3 °C) Modified atmosphere packaging (elevated CO_2, reduced O_2)

1.8.1. Physical approaches to controlling pathogenic and spoilage organisms on fresh meat and poultry.

Refrigeration is the most important method of preservation for the meat industry whereby fresh food products are held at low temperatures (0-4 °C) to reduce or inhibit microbial growth. However, even under refrigerated temperatures, meat continues to spoil due to the activity of aerobic psychrotrophs. Additionally, the psychrotrophic pathogen *L. monocytogenes* will grow at temperatures approaching 0 °C (Klima and Montville, 1995) and the growth of *Salmonella* in fresh meats has been reported at temperatures as low as 2 °C (D'Aoust, 1991).

A possible method for the control of microbial growth on raw meats and poultry is the use of irradiation, which has been legally allowed in 36 countries since 1989 (Loaharanu, 1989) and has been approved for many food types including many vegetables, pulses, poultry, fish and seafood (Urbain, 1978). The WHO has approved as safe, the exposure of foods to radiation doses up to 7 kGy (Jay, 1996). Irradiation is considered to be the only method capable of decontaminating both the surface and deep muscle of fresh meat and poultry. There is a substantial literature on the effects of irradiation on spoilage and pathogenic microorganisms in foods. The dose levels required vary with the nature of the foodstuff, the target microorganism(s) and the temperature at which irradiation is carried out. Gamma irradiation has been shown to be effective in controlling levels of: *C. jejuni* in poultry (Lambert and Maxcy, 1984) and ground beef (Clavero *et al.*, 1994); *L. monocytogenes* in beef (Thayer and Boyd, 1995a); and *S. typhimurium* in mechanically deboned chicken (Thayer *et al.*, 1995b). However, a major barrier to the increased use of irradiation in food treatments is a widely held but unsubstantiated view amongst the public that food irradiation is inherently unsafe and undesirable. There is also some scientific evidence that irradiation may reduce the nutritional value of some foods by destruction of aromatic amino acids (Johnson and Moser, 1967) and vitamins, particularly B vitamins (Liuzzo *et al.*, 1966), as well as producing rancidity and off odours (Wick *et al.*, 1967) Nevertheless, the increasing incidence of foodborne disease may stimulate the use of

irradiation in the food industry (Monk *et al.,* 1995). It might also reduce costs by extending the shelf life of foods, particularly fresh meat products.

Spray washing of carcasses in hot water during processing has been demonstrated to improve the microbiological quality of meat by the physical removal of microorganisms from the surface (Crouse *et al.,* 1988). The efficacy of this decontamination approach is dependent on the temperature of the spray water, the line pressure, volume of water per carcass and the speed at which carcasses (or products) travel through the spray treatment (Anderson *et al.,* 1975; Kelly *et al.,* 1981). The procedure is applicable to food processing environments and allows the possibility of additional interventions, for example, by chemical treatments which might be particularly important in controlling cross-contamination of carcasses.

Other physical approaches to reducing microbial loads on foods include the use of high pressures and modified atmosphere packaging. The latter is already widely used and alters the microbial flora rather than eliminating spoilage organisms (Lambert *et al.,* 1991). Experimentally, the use of high pressures has been shown to potentially inactivate pathogenic microorganisms when used in combination with additives like lysozyme and nisin (Hauben *et al.,* 1996). However, there are no reported current commercial applications.

1.8.2. Decontamination of fresh meat and poultry by chemical treatments.

The principle chemical treatments used or proposed for use in the decontamination of fresh meat and poultry are those involving chlorine, phosphates, quaternary ammonium compounds and low molecular weight organic acids.

1.8.2.1. The use of chlorine treatments.

Chlorine is presently used as a disinfectant in the food industry for food contact surfaces and utensils. Several studies have reported the efficacy of chlorine as a chemical decontaminant of meat and poultry. Currently, the highest legally allowed level of chlorine for use as a food decontaminant in the US is 200 ppm free chlorine (James *et al.*, 1997). Kelly *et al.* (1981) reported that spraying lamb carcasses with a mild hypochlorite solution (30-50 ppm available chlorine) significantly reduced the microbial population in comparison to spraying carcasses with hot water; the duration of spraying was considered an important factor in reducing bacterial numbers. Similarly, Skelley *et al.* (1985) found that exposure to a hypochlorite solution containing 450 ppm available chlorine reduced bacterial populations on pork carcasses. However, Cutter and Siragusa (1995a) reported that the spray application of hypochlorite solutions containing up to 800 ppm available chlorine reduced *E. coli* populations on beef by less than 1.3 $\log_{10}$ cycles.

The efficacy of chlorine dioxide as a terminal decontaminant has been investigated in relation to both meats and poultry. Chlorine dioxide has a greater oxidising capacity than chlorine and was found to reduce bacterial populations both in poultry chill water and on poultry carcasses (Berg *et al.*, 1986). However, Cutter and Dorsa (1995) demonstrated that spray treatments of beef with chlorine dioxide were no more effective than water spraying in reducing the microbial population. Salmide, a sodium chlorite-based oxyhalogen disinfectant has been reported to give high kills (4.1 $\log_{10}$ reductions) of suspended cells of *Salmonella* spp. (Mullerat *et al.*, 1995).

1.8.2.2. The use of phosphate treatments.

Phosphates are frequently used in the food industry as processing aids or additives. In the meat industry, phosphates are mainly used to retain moisture. However, they have recently been evaluated for their antimicrobial properties. Poultry carcasses chilled in sodium tripolyphosphate (STPP) and sodium pyrophosphate had an increased shelf-life

(Spencer and Smith, 1962; Elliot *et al.*, 1964) and the growth of *Aeromonas hydrophila* on ground beef at refrigerated temperatures was inhibited by a polyphosphate plus NaCl combined treatment, when compared to individual treatments with polyphosphates or NaCl alone (Palumbo *et al.*, 1995). Bekaplus MSP, a blend of sodium polyphosphate, sodium orthophosphate and sodium metaphosphate is also reported to have a significant effect in controlling *E. coli* O157:H7 growth in fresh pork sausage (Flores *et al,*, 1996). However, it was not effective in preventing the growth of *E. coli* O157:H7 in ground beef or of *L. monocytogenes* and *S. typhimurium* in various fresh meat products (Flores *et al.*, 1996). Brifisol K, a commercial blend of sodium acid pyrophosphate and orthophosphoric acid, has been reported to significantly reduce *E. coli* on postchill broilers and extend product shelf-life; however, it was recommended that it be used with a surfactant (unspecified), to aid detachment of surface microorganisms, and in conjunction with a chill water treatment, to rapidly cool carcasses during processing (Rathgeber and Waldroup, 1995).

Recently trisodium phosphate (TSP) has been approved for use as a post-chill processing treatment for the reduction of microorganisms on raw poultry carcasses (Giese, 1992). Significant reductions in Gram -ve bacterial populations on carcasses were observed but only using high TSP concentrations (8 - 12 % w/v; Bender and Brotsky, 1991). At these concentrations, the pH of TSP solutions is >12. However, it has been speculated that an important factor in the effectiveness of TSP is its role as a surfactant in aiding bacterial removal from fatty surfaces (Giese, 1993). A detailed discussion of the literature relating to TSP-treatments for killing microorganisms on foods is given in Section 4.

1.8.2.3. The use of quaternary ammonium compounds.

Quaternary ammonium compounds (QACs) are presently used as disinfectants in the food industry for food contact surfaces and utensils. Recently, there have been preliminary investigations into the use of such surface-active agents in reducing bacterial attachment to meat and poultry. Breen *et al.* (1995) studied a series of QACs and showed that, at low

concentrations (0.1 % w/v), cetylpyridinium chloride was able to prevent attachment of viable *S. typhimurium* cells to poultry tissue. The authors claim that the efficacy of QAC at such low concentrations (0.1 % w/v) might make them more suitable than trisodium phosphate TSP (used at approximately 10 % w/v; see Section 4) in the decontamination of poultry carcasses. However, it appears that the possible presence of toxic QAC residues in chicken products has not been considered.

1.8.2.4. The use of organic acids.

Low molecular weight organic acids are widely used in the food industry as preservatives. Their effects are due to a reduction in both extracellular and intracellular pH. Their ability to affect intracellular pH is due to their low molecular weight and thus, their relatively high rate of uptake by cells when in an uncharged (undissociated) form (Lambert *et al.*, 1991). Recently, there has been interest in using organic acids to decontaminate meat and poultry. The organic acids tested have included acetic (Dickson, 1992; Dickson and Siragusa, 1994), gluconic (Zepeda *et al.*, 1994), lactic (Greer and Dilts, 1995) and propionic acids (Golden *et al.*, 1995). Acetic and lactic acids have been most thoroughly evaluated.

Lactic acid is a natural product of sugar fermentation by lactic acid bacteria. It has low toxicity, GRAS (Generally Regarded As Safe) status and is approved by the US Food and Drug Administration (FDA) as a food additive. There is no general specified maximum concentration limit for its use, but in meat products the concentration should be the lowest consistent with its intended purpose (Anon, 1993a). Lactic acid has been successfully demonstrated to reduce the numbers of various microorganisms present on the surface of pork, chicken and beef (e.g. van Netten *et al.*, 1995; Zeitoun *et al.*, 1994; Kotula *et al.*, 1994). Additionally, it had a bacteriostatic effect on Gram-negative psychrotrophs, suggesting that it might prolong the shelf-life of refrigerated products (Zeitoun *et al.*, 1994). However, concentrations of lactic acid that successfully

decontaminate meat and poultry appear to cause product discoloration (Izat *et al.,* 1989) and affect flavour (Smulders *et al.,* 1986).

Acetic acid also has low toxicity, GRAS status and is approved by the US FDA. The maximum amount allowed to be incorporated into meat products is either 0.6% or, when used to alter the acidity or as a surface treatment, the lowest concentration necessary for the intended purpose (Anon, 1993b). Dickson (1992) and Dickson and Siragusa (1994), have demonstrated the efficacy of acetic acid (at 1 and 2 % v/v respectively) against a range of foodborne pathogens, including S. *typhimurium*, *E. coli* O157 and *L. monocytogenes*, attached to beef muscle and fat. Thus, some organic acids may be useful in meat and poultry decontamination. However, the low pH of organic acid treatment fluids necessary for cell killing may produce unacceptable colour changes in the product and might also corrode processing equipment. Therefore, it is likely that in any commercial application, they will be combined with other treatments (Hwang and Beuchat, 1995; Monk *et al.,* 1996).

1.8.2.5. The use of natural antimicrobial substances in the decontamination of fresh meat and poultry.

A number of chemicals, and particularly hypochlorite, are successful in reducing the contamination of processing water. However, they are generally less suitable for the decontamination of fresh meat and poultry (Section 1.8.2.1). They may leave toxic residues, change the organoleptic properties and surface appearance of the product and frequently involve high costs (Slavik *et al.,* 1991). In addition, there is consumer pressure for more 'natural' foods which have been subjected to minimal processing and contact with synthetic chemicals. Thus, there is significant interest within the food industry in developing treatment processes and preservation procedures based on natural antimicrobial compounds. The most promising of these compounds in relation to the preservation of meat and poultry are lysozyme and bacteriocins, of which nisin is the only example currently available commercially. The potential application of lysozyme and

nisin is considered below. Lactoperoxidase, a natural enzyme of milk, in combination with added potassium thiocyanate or sodium iodide and hydrogen peroxide (Wolfson *et al.*, 1994; Bianchi *et al.*, 1994) has also been proposed as a means of decontaminating poultry. In milk, the lactoperoxidase system can be readily utilised by simply adding the hydrogen peroxide and thiocyanate required (Wolfson *et al.*, 1994).

1.8.2.5.1. Lysozyme.

Lysozyme is a natural antimicrobial enzyme present in many body fluids, such as tears and saliva, and is also found in milk and eggs. Hen egg white lysozyme is composed of a single polypeptide chain with a molecular weight of 14,600 Da. It is heat labile under alkali conditions, and at pH 8, activity is lost by heating for 3 min at 65°C. However, there was little reduction in activity when heated at 100°C for 30 min at acid pH (pH 5) (Ahern and Klibanov, 1985). The enzyme is also stable when stored dry at 5 °C (Salton, 1957). The stability of the enzyme has been attributed to the presence of four disulphide bridges that cross-link the polypeptide chain (Jolles *et al.*, 1963).

Lysozyme acts specifically against the peptidoglycan component of bacterial cell walls. Peptidoglycan is composed of a backbone of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) residues, linked by β(1-4) glycosidic linkages and containing short peptide chains attached to the NAM ring (Fig 1.3). Cross-linking of the peptides between adjacent polysaccharide chains confers mechanical rigidity (Fig 1.4). Lysozyme hydrolyses the bond between NAM and NAG and in consequence, the rigidity of the peptidoglycan layer of the bacterial cell wall is reduced, leading to cell lysis.

Lysozyme is able to gain direct access to the peptidoglycan of most Gram-positive bacteria, as peptidoglycan is distributed throughout the cell wall and may constitute up to 70 % of the cell wall dry weight (Schlegel, 1990). However, in Gram-negative bacteria, it comprises only approximately 10 % of the cell wall dry weight and occurs as a discrete layer, protected by an outer lipid membrane (Fig. 1.5). The detailed structure of the cell

wall in Gram -ve and Gram +ve bacteria is shown in Figs 1.5 and 1.6. The outer lipid membrane of Gram -ve bacteria is permeable to small molecules (<500 Da), through pores created by porin proteins. However, larger molecules are excluded unless specific proteins for their uptake into the periplasmic space are present. Lysozyme is clearly too large a molecule to ordinarily cross the outer membrane and is therefore generally ineffective against Gram -ve cells.

Hen egg white lysozyme has GRAS status (Owen Fields, 1996). It is approved for food use in Europe (Hughey and Johnson, 1987) and is widely used, throughout the world, as an antibacterial agent in the food industry. A major use is in cheese production, where it prevents gas formation and spoilage caused by the growth of clostridial species (Bester and Lombard, 1990); it also shows activity against Gram +ve pathogenic foodborne bacteria such as *Bacillus* and *Listeria* (Hughey and Johnson, 1987). It has been the aim of many studies to extend the antimicrobial spectrum of lysozyme, particularly to include Gram-negative bacteria. This could be achieved by disruption of the outer cell membrane, which may be brought about by such treatments as: heat (Becker and Hartsell, 1954); freezing and thawing (Kohn, 1960); extraction of lipopolysaccharide from the outer face of the outer membrane with lipid solvents or alkali (Becker and Hartsell, 1955); starvation at extreme pH (Grula and Hartsell, 1957); and treatments with detergents (Colobert, 1957) or polybasic antibiotics (Warren *et al.*, 1957).

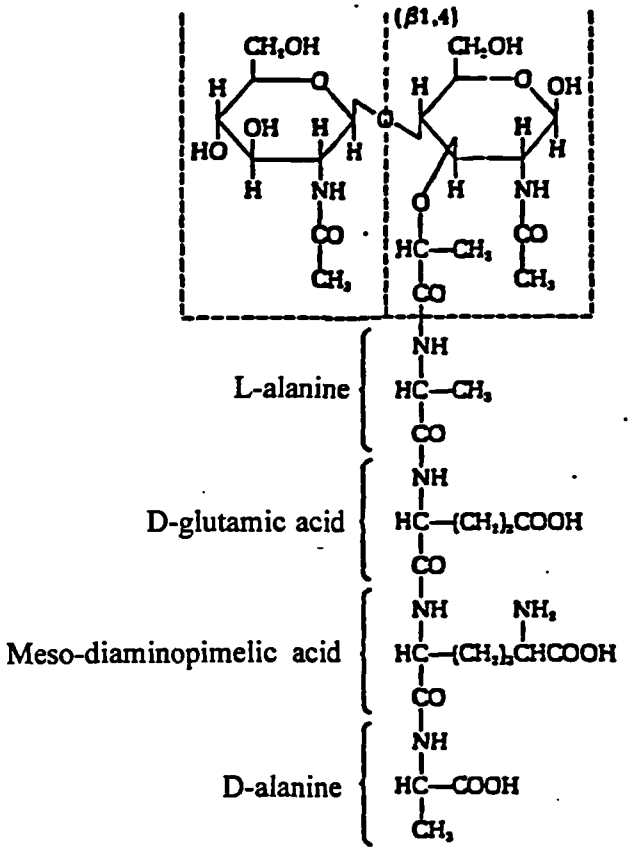


Fig. 1.3. The basic repeating unit of the polysaccharide chain of peptidoglycan with attached peptide. The amino acids in the peptide chain may vary between species.

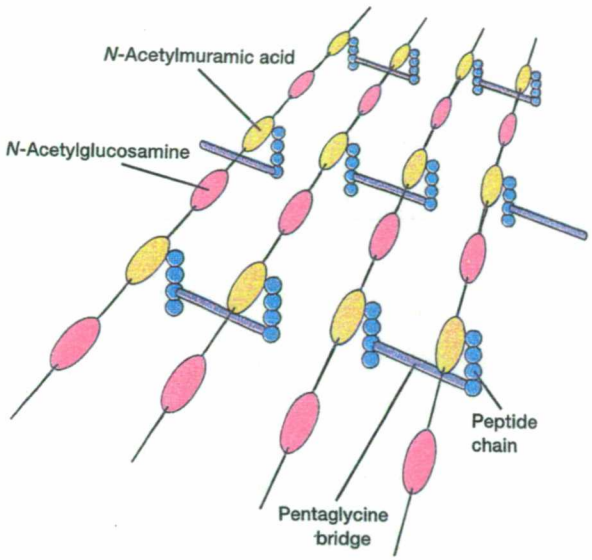


Fig.1.4. The cross linking of the polysaccharide chains of peptidoglycan. Cross linking may be regular (as shown) and involve peptide bridges (e.g. the pentaglycine bridge of *Staph. aureus*); or it may be irregular with direct cross linking of tetrapeptide chains (between the third and fourth amino acids). (Adapted from Prescott *et al.*, 1993.)

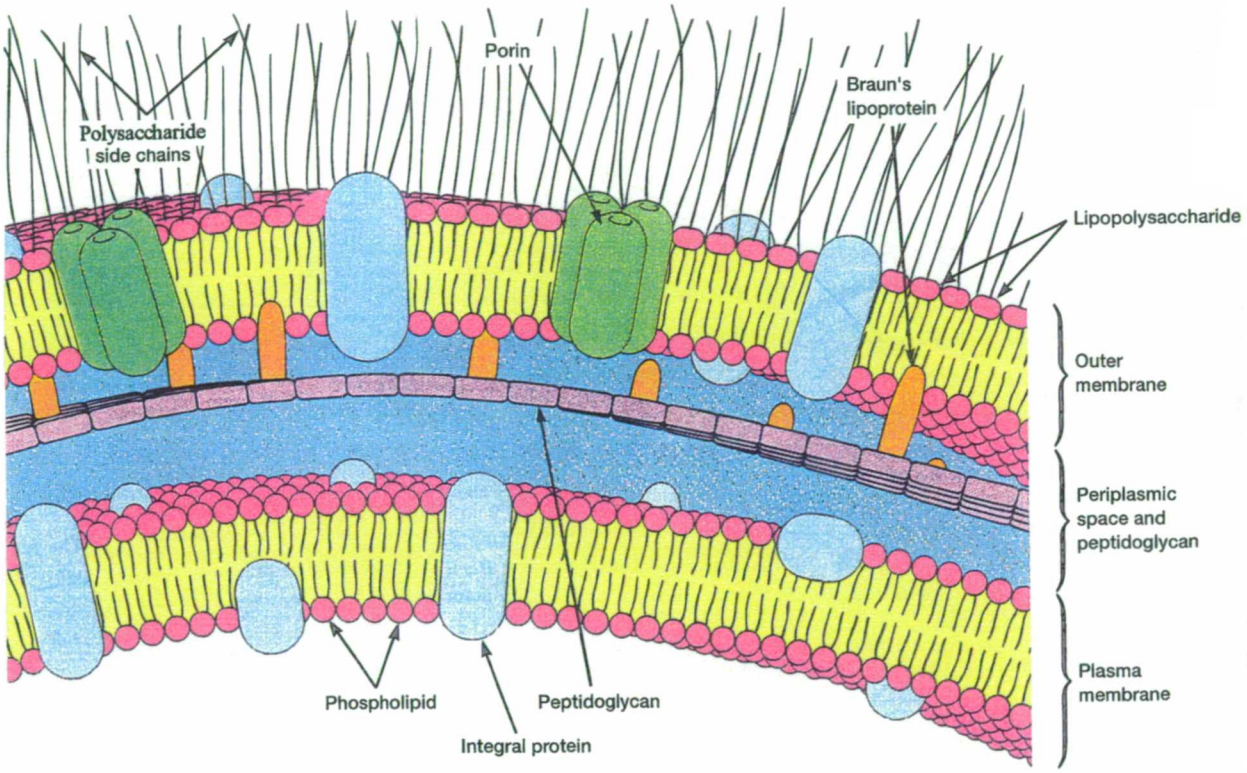


Fig.1.5. Cell wall of Gram-negative bacteria.

(Adapted from Prescott *et al.*, 1993.)

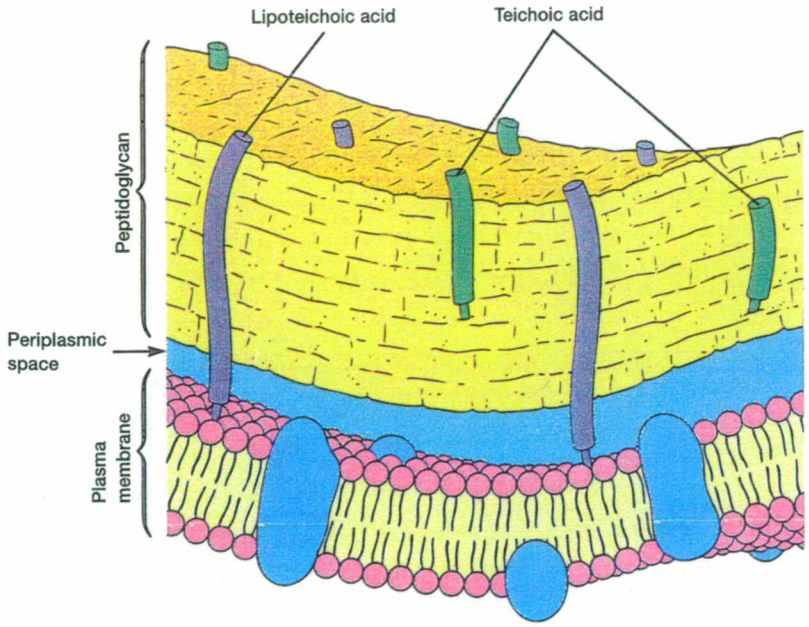


Fig.1.6. Cell wall of Gram-positive bacteria.

(Adapted from Prescott *et al.*, 1993.)

The chelating agent EDTA has also been shown to increase the susceptibility of Gram -ve bacteria to lysozyme (Repaske, 1958) ; and in more recent studies, Hughey and Johnson (1987) demonstrated that it induced susceptibility in a number of Gram-positive and Gram-negative pathogens, isolated from food poisoning outbreaks. In Gram -ve bacteria, EDTA may specifically disrupt the polysaccharide component of the lipopolysaccharide of the outer membrane (see Fig1.5; Stevens *et al.,* 1992). The susceptibility of *E. coli* cells to lysozyme was increased when EDTA treatment was combined with hypo-osmotic shock (Witholt *et al.,* 1976). After 3.5 min incubation in a Tris-EDTA buffer containing lysozyme and sucrose, dilution of the cell suspension in distilled water resulted in cell lysis. However, EDTA appears unexploited for use in the poultry processing industry. More recently, Chatzopolou and Miles (1992) and Chatzopolou *et al.* (1993) were able to increase the susceptibility of *Salmonella* and other Gram-negative microorganisms to lysozyme using a two-step osmotic shock procedure. In the first step, cells (in suspension, or attached to chicken skin) were exposed to a hyper-osmotic NaCl solution. This was followed, in the second step, by hypo-osmotic shock using distilled water containing lysozyme. It appeared that lysozyme was able to penetrate the outer membrane during the large flux of water into cells that accompanied their transfer to the hypo-osmotic solution. The optimal procedures developed are described in Section 4.2. The procedures were highly effective against suspended cells, but considerably less effective against cells attached to food surfaces.

1.8.2.5.2. Bacteriocins.

Bacteriocins are antimicrobial peptides, many of which are produced by lactic acid bacteria. They vary greatly in chemical nature, mode of action and host specificity. The most widely studied bacteriocin is nisin A, a cationic polypeptide of thirty four amino acids (3353 Da) produced by *Lactococcus lactis*. It disrupts the cytoplasmic membrane of susceptible species, dissipating the proton motive force and causing pore formation and efflux of low molecular weight solutes (Abu-Amero *et al.*, 1996). It is active against the majority of Gram +ve bacteria. However, Gram -ve organisms are resistant unless the outer membrane of the wall is damaged by, for example, chelating agents (Cutter and Siragusa, 1995b, 1995c).

Nisin is naturally produced by *Lactoc. lactis* in many fermented foods. As a food additive, it has GRAS status (Owen Fields, 1996) and has been approved for various food applications in more than forty countries (Gould, 1996). It has found particular application in the prevention of late-blowing of cheese due to growth of *Clostridium* spores (Daeschel, 1989) and preventing the outgrowth from germinated spores of *Cl. botulinum* present in pasteurised processed cheese spreads (Delves-Broughton, 1990). In addition, most European countries have approved the use of nisin, in canned vegetables and other foods, to kill *Cl. botulinum*. Nisin is also effective against other foodborne pathogens including *L. monocytogenes* and *Staph. aureus*.

The fact that bacteriocins, such as nisin, are 'natural' may promote their acceptability amongst the public, although they might be perceived as being 'antibiotics'. It may be possible, in certain foods, to overcome such difficulties by the use of a 'food-grade' *Lactobacillus* producer strain, for which specific product-labelling would not be required (Holzapfel *et al.*, 1995).

1.9. Aims of the Thesis.

The surface contamination of many food products, by both food spoilage and potentially pathogenic microorganisms, is to some extent unavoidable. For example, animal carcasses are almost invariably contaminated with faecal material (Ayres, 1955). There is also a risk that fresh and processed foodstuffs might become contaminated during distribution and subsequent handling, either from other contaminated foodstuffs, food handlers or by contact with contaminated surfaces. The principal surfaces in this context are food preparation surfaces in commercial and domestic environments, where surfaces have previously been in contact with contaminated foodstuffs.

This thesis is concerned primarily with the surface contamination of foodstuffs and food contact surfaces. Two major aspects have been considered. These are the survival of food spoilage and potentially pathogenic microorganisms on typical food preparation surfaces; and the development of novel methods for decontaminating surfaces. These methods were developed particularly in relation to the decontamination of chicken skin, though some work was also conducted using inert surfaces.

In determining the survival of bacteria on surfaces, a major consideration was the efficiency of surface sampling methods. Thus, initially a novel method for enumerating viable microorganisms on surfaces was developed. In this method, described as an *'in situ'* method, surfaces were overlaid with agar and, after incubation, viable organisms able to form colonies were detected by reaction with a metabolic dye (nitroblue tetrazolium). The method was successfully used to investigate the effect of a variety of factors on the survival of bacteria inoculated onto test surfaces.

The factors investigated were:

(i) surface type

(ii) organism type

(iii) nature of the suspending fluid (in which organisms were applied to test surfaces)

(iv) inoculum density

(v) drying time (i.e. time after application to surface)

(vi) relative humidity

(vii) temperature

The novel method for surface decontamination arose out of work previously conducted at King's College. In this work (Chatzopoulou, 1991; Chatzopoulou *et al.*, 1993; Chatzopoulou and Miles, 1992), it was shown that the susceptibility of Gram -ve cells to lysozyme could be markedly enhanced if cells were subjected to osmotic shock. The optimum procedure involved the treatment of cells (in suspension or attached to chicken skin or meat) with 0.8 M NaCl, followed by dilution in deionised water containing a low concentration (10-100 $\mu g\ ml^{-1}$) of lysozyme. However, although the method worked well for suspended cells, it was less effective for attached cells. Subsequent to the work of Chatzopoulou (1991), a patent was published in which the use of TSP to decontaminate chicken carcasses was proposed (Bender and Brotsky, 1991). Unfortunately, the method was only successful for Gram -ve bacteria and required very high TSP concentrations (10% w/v, pH >11). It was later suggested that the reduction in bacterial cell counts was in part due to the surfactant effect of TSP, which aided the detachment of cells from the skin surface (Lillard, 1994). If TSP was able to act as a surfactant, it was possible that it might disrupt the outer cell membrane of Gram -ve bacteria, thus enhancing the killing effect of osmotic shock and/or lysozyme treatments. Therefore, the effect of combining these killing treatments with exposure to TSP was investigated using a variety of Gram +ve and Gram -ve bacteria associated with food spoilage or foodborne disease. Experiments were conducted using suspended cells, in the presence and absence of high organic loads, and cells attached to food contact

surfaces, chicken skin and the surfaces of other foodstuffs. A major factor in achieving high kills was TSP concentration which was optimised for the various experimental conditions. The effectiveness of TSP-killing methods in decontaminating chicken skin was also demonstrated using the *in situ* nitroblue tetrazolium reduction method, referred to above.

SECTION II.

Materials and Methods

This section details the general methods and experimental procedures undertaken during the course of this thesis.

2.1. Organisms.

Campylobacter jejuni (NCTC 11626), *Listeria monocytogenes* (NCTC 7973), *Pseudomonas fluorescens* (NCTC 10038), *Salmonella enteritidis* (NCTC 6676), *Escherichia coli* (NCTC 12241) and *Staphylococcus aureus* (NCTC 8532) were obtained as freeze-dried cultures from the National Collection of Type Cultures (NCTC), Colindale, UK. *Escherichia coli* (NCIMB 948) was obtained from the National Collection of Industrial and Marine Bacteria Ltd. (NCIMB), Aberdeen, Scotland. *Staphylococcus aureus* (C23) was obtained from the Division of Life Sciences Culture Collection, King's College London. *Salmonella enteritidis* phage type 4 (strain 5159648), a recent non-motile clinical isolate, was kindly provided as an agar culture by Dr. Linda Ward, Laboratory of Enteric Pathogens, Public Health Laboratory Service, Colindale, UK. *Salmonella enteritidis* strain PT4 (5159648) was used only in experiments on the decontamination of food preparation surfaces (ceramic and stainless steel); in all other experiments, the *Salmonella* strain used was NCTC 6676.

2.2. Media for bacterial growth.

2.2.1. Brain Heart Infusion Broth.

Brain heart infusion (BHI) broth was used for the batch culture of all organisms except *C. jejuni*. It was prepared by reconstituting brain heart infusion powder (Oxoid, Basingstoke, UK; 37 gL^{-1}) in distilled water. The broth was autoclaved in 25 ml quantities in 250 ml conical flasks at 121 °C, 15 psi for 15 min.

2.2.2. Enriched Brain Heart Infusion Broth.

Enriched brain heart infusion was used for the growth of *C. jejuni.* Brain heart infusion broth powder (Oxoid; 37 g) and yeast extract (Oxoid; 2.5 g) were added to 1 L distilled water. The broth was autoclaved at 121 °C, 15 psi for 15 min and after cooling to a temperature of below 45 °C, 10 % v/v inactivated horse serum (Gibco BRL, Paisley, Scotland) was added. The complete medium was dispensed in 25 ml quantities in 70 ml cell culture flasks (Sterilin, Staffs, UK).

2.2.3. Nutrient Agar.

Solid medium for the growth and maintenance of all organisms except *C. jejuni,* was prepared by reconstituting nutrient agar (Oxoid; 28 gL^{-1}) in distilled water and autoclaving the suspension at 121 °C, 15 psi for 15 min. When the agar had cooled to a temperature of 50 °C, 15 ml quantities were dispensed into 9 cm diameter sterile plastic petri dishes (Sterilin). Once the agar had solidified, the plates were stored at 4 °C for up to 2 weeks.

2.2.4. Blood Agar.

Blood agar was used for the growth and maintenance of *C. jejuni.* It was prepared by boiling blood agar base No.2 (Oxoid; 40 gL^{-1}) in distilled water and autoclaving the suspension at 121 °C, 15 psi for 15 min. When the autoclaved mixture had cooled to a temperature of between 45 and 50 °C, 7 % v/v horse blood (Laked; Oxoid) was added. The suspension was mixed by gentle agitation to minimise foaming. Aliquots (approximately 15 ml) were then dispensed into 9 cm diameter sterile plastic petri dishes (Sterilin). Once the agar had solidified, the plates were stored at 4 °C for up to 2 weeks.

2.2.5. Gelatin Agar.

The composition of gelatin agar was:

Lab Lemco Powder (Oxoid)	1.0 g
Yeast extract (Oxoid)	2.0 g
Peptone (Oxoid)	5.0 g
Sodium chloride (Sigma, Poole, UK)	5.0 g
Gelatine (Merck, Germany)	160 g
Deionised Water	to 1.0 L

The medium was autoclaved at 121 °C, 15 psi for 15 min. When the agar had cooled to a temperature of 50 °C, 15 ml aliquots were dispensed into 9 cm diameter sterile plastic petri dishes (Sterilin). Once the agar had solidified, the plates were stored at 4 °C for up to 1 week.

2.3. Growth of organisms.

Listeria monocytogenes, *E. coli* and *Staph. aureus* were grown aerobically at 37 °C in BHI broth or on nutrient agar (NA) plates. *Pseudomonas fluorescens* was similarly grown, but at 30 °C. For liquid cultures, 250 ml flasks containing 25 ml medium were inoculated with 1 ml of a fully grown culture (18 h) or colonies from agar plates. Flasks were incubated on a shaking incubator (Gallenkamp, Fisher Scientific, Leicester, UK) at 200 rpm. *Campylobacter jejuni* was grown statically in 70 ml cell culture flasks (Sterilin) containing 25 ml enriched BHI broth or on blood agar plates. Cultures were incubated at 37 °C in an atmosphere containing 10 % (v/v) CO_2 and 10 % (v/v) O_2. The modified atmosphere was obtained using a disposable gas generating kit (Oxoid BR60) in conjunction with an anaerobic jar (Oxoid, AnaeroJar).

2.4. Maintenance of organisms.

Long term storage of cultures was carried out at -70 °C. Glycerol (cryoprotectant; final concentration 15 % v/v) was added to early stationary phase cultures (see section 2.8.1.) which were dispensed (1 ml aliquots) aseptically into sterile cryotubes (Sigma) and frozen at -70 °C. Bacterial cultures were also stored on agar plates at 4 °C for up to 14 days.

2.5. Cell suspension media.

2.5.1. Quarter strength Ringers solution.

Quarter strength Ringer's solution was prepared by dissolving one Ringer tablet (Oxoid) in 500 ml deionised water. The solution was autoclaved at 121 °C, 15 psi for 15 min. The composition of quarter strength Ringer's solution was:

Sodium chloride	2.25 gL^{-1}
Potassium chloride	0.105 gL^{-1}
Calcium chloride	0.12 gL^{-1}
Sodium bicarbonate	0.05 gL^{-1}

The solution had a final pH value of approximately pH 7.0.

2.5.2. Swab Resuscitation Medium.

The composition of swab resuscitation medium, as described by Humphrey *et al.* (1995), was:

Peptone (Oxoid)	1.0 g
Sodium chloride (Sigma)	8.5 g
Sodium thiosulphate (BDH, Poole, UK)	3.0 g
Tween 80 (Sigma)	30 ml
Deionised water	970 ml

The pH was adjusted to 7.1 ± 0.1 using 0.1 M sodium hydroxide or 0.1 M hydrochloric acid. The medium was then autoclaved at 121 °C, 15 psi for 15 min.

2.6. Chemicals, antibacterial agents and detergents.

All chemicals used in this study were obtained from Sigma, BDH, or Merck. The source and preparation of a number of specific reagents is described in the following sections.

2.6.1. Nitroblue tetrazolium.

Nitroblue tetrazolium (NBT; 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride; Sigma) was prepared by dissolving NBT (1 gL^{-1}) in deionised water. The NBT solution was sterilised by membrane filtration (membrane filter pore size 0.2 µm; Gelman Sciences, USA). NBT solutions were stored at 4 °C for up to 2 weeks.

2.6.2. Lysozyme.

Hen egg lysozyme (Sigma, L-6876) was prepared by dissolving lyophilised lysozyme crystals (10-100 $\mu g\ ml^{-1}$) in deionised water. The lysozyme solution was sterilised by membrane filtration (membrane filter pore size 0.2 μm; Gelman Sciences). Lysozyme solutions were freshly prepared prior to each experiment.

2.6.3. Nisin.

Nisin (freeze-dried form comprising 2.5 % nisin with sodium chloride and denatured milk solids; Sigma) was dissolved in deionised water and stored frozen at -70 °C as a 3 mM stock solution. When required for use, the stock solution was thawed and sterilised by membrane filtration (membrane filter pore size 0.2 μm; Gelman Sciences).

2.6.4. Detergents.

A number of test detergents were used in experiments to decontaminate surfaces. These were:

Liquid Multiclean SU 125 (Unilever Industrial, Runcorn, UK). Liquid Multiclean SU 125 is a general purpose liquid detergent for use in cleaning hard surfaces in food, beverage and associated industries. It contains EDTA and anionic surfactants (to remove fatty and proteinaceous soiling) and is recommended for use at 1-5 % w/w. Liquid Multiclean was used in surface decontamination experiments at a concentration of 2 % v/v.

Quatdet SU321 (Unilever Industrial). Quatdet SU321 is a liquid industrial sanitiser developed for use as a disinfectant or detergent disinfectant on hard surfaces in food, beverage and associated industries. It contains cationic and nonionic surfactants with

trisodium nitrile tetraacetic acid and tetrasodium EDTA to give good soil penetration. Quatdet has been demonstrated to be effective against a wide range of microorganisms (Unilever Industrial product information sheet, 1996). For manual applications it is recommended for use at 1 % w/w. Quatdet was used in decontamination experiments at a concentration of 1 % v/v.

Liquid Multiclean SU 125 and Quatdet SU321 were kindly provided by Dr. John Regarlsford on behalf of Unilever Industrial, Runcorn, UK.

Fairy Liquid (Proctor and Gamble, Newcastle, UK). Fairy Liquid is a household surfactant for use as a dish detergent in domestic kitchens. It contains amphoteric, nonionic and anionic surfactants and was used in decontamination experiments at a concentration of 2 % v/v.

2.7. Test surfaces.

The test surfaces were ceramic tile (BS 6431: Part 9), glass (borosilicate), plastic (crystal polystyrene BS611: Part 2 1990), stainless steel sheet type 316 (BS1449: Part 2 316 S11), stainless steel sheet type 304 2B (BS 1449: Part 2 304 S31; semi-bright grey surface) and stainless steel sheet type 304 240Si (BS 1449: Part 2 304 S31 unidirectional finish obtained by abrasion with 240 grid silicone carbide). The glass and plastic surfaces were the base of 9 cm diameter petri-dishes (Pyrex and Sterilin respectively, obtained from Fisher Scientific, UK). Other test surfaces were squares (9 x 9 cm) cut from 1 mm thick stainless steel sheet type 316 (Rightons, High Wycombe, UK) or stainless steel sheet types 304 2B and 304 240Si (kindly provided by Dr. David Dulieu, Avesta Sheffield, Sheffield, UK) and glazed and abraded ceramic wall tiles (kindly provided by H.R. Johnson Tiles Ltd., Stoke on Trent, UK). Abraded tiles had been prepared using the protocols specified by the Porcelain and Enamels Institute. The protocols required the use of a number of steel ball bearings of differing diameters, a coarse alumina powder and a quantity of water. These items were placed in a cylinder

which had an open end in contact with the glazed surface of the tile. The cylinder was gyrated mechanically for 1500 cycles. Ceramic, glass and stainless steel test surfaces were sterilised overnight in an air-circulating oven at 160 °C. Plastic surfaces (disposable petri dishes) were obtained sterile.

2.8. Bacterial survival on food contact surfaces.

2.8.1. Inoculation of test surfaces.

Overnight broth cultures of *L. monocytogenes*, *P. fluorescens*, *S. enteritidis*, *E. coli* and *Staph. aureus* were diluted 1/25 in fresh BHI broth and grown, on a shaking incubator (Section 2.3) until the early stationary phase (approximately 4 h incubation). Early stationary phase cells are generally regarded as the most resistant to stresses associated with drying (Strange and Cox, 1976). To ensure the stationary phase had been reached, culture optical density (OD) was monitored using a Corning spectrophotometer (Model 258, Ciba Corning Diagnostics, Halstead, UK) at 600 nm, until a constant and maximal value had been reached. This value was determined for each organism in control experiments. Aliquots (1.5 ml) of early stationary phase cultures, i.e. cultures that had just reached the maximal OD value, were washed three times in sterile deionised water and resuspended in 10 ml of test diluent. The test diluents used were: sterile deionised water; quarter strength Ringer solution (Oxoid); brain heart infusion broth (Oxoid); 10 % serum (inactivated new-born calf, Gibco); and solutions of sucrose (0.1-10 % w/v) or NaCl (0.1-10 % w/v). Ten-fold serial dilutions of cell suspensions were prepared (using the diluent in which cells were suspended) and 20 µl drops of diluted cell suspensions were then inoculated onto test surfaces. The test surfaces were the base of glass or plastic petri dishes (9 cm diameter) or squares (9 cm^2) of stainless steel sheet or ceramic tile enclosed in 12 cm diameter glass petri dishes. Descriptions of these test surfaces are given in Section 2.7. In typical experiments, a minimum of ten drops were inoculated on duplicate test surfaces for each dilution of the cell suspension tested.

2.8.2. Incubation of test surfaces.

Drops of bacterial suspensions on test surfaces were dried by incubating the dishes at a controlled temperature which was 37 °C, unless otherwise stated. Metal grids (previously heat sterilised) were inserted between the base and lid of petri dishes enclosing the test surfaces, to facilitate drying. The relative humidity (RH) was between 38 and 47 % for the experiments reported, except where the effect of RH on cell survival was specifically investigated. For these experiments, RH was controlled using a Stuart Scientific Incubator (S.I. 60; Surrey, UK) with forced air circulation over a tray (30 cm x 50 cm) containing silica gel, NaCl-water (250 gL^{-1}; Winston and Bates, 1960) or water (500 ml). RH values of approximately 30, 50 and 80 % were readily achieved in this way and during the course of experiments (up to 3 h) there was little (± 2 %) fluctuation in measured RH values.

2.8.3. Detection of bacterial survival by an *'in situ'* method.

Nutrient agar (46 °C) was carefully poured over previously inoculated surfaces (enclosed within petri dishes) to a depth of 3 mm. The plates were then incubated as described in Section 2.3. for up to 48 h. NBT (1.0 gl^{-1}, filter sterilised) was pipetted onto plates (1 ml per 20 ml solidified agar) and plates were incubated at room temperature for a minimum of 6 h to allow colour development (deep blue) of colonies at the agar-test surface interface.

2.8.4. Detection of bacterial survival by swabbing method.

To recover organisms dried on surfaces by swabbing, swabs (sterile, non-absorbent cotton wool; Phillip Harris, Lichfield, UK), were moistened in 10 ml swab resuscitation medium (SRM; Section 2.5.2) and moved over the surface using a standardised procedure (Sveum *et al.*, 1992). Swabbing was repeated using a dry swab and both swabs were then broken into the recovery medium, which was vortexed for 10 sec. The medium and appropriate dilutions, also in SRM, were plated on nutrient agar (10 x 20 µl drops per plate, in triplicate). After drops had dried, plates were incubated as described in Section 2.3.

2.9. Bacterial decontamination of food-preparation surfaces.

Surfaces (ceramic, stainless steel types 316) were inoculated with *S. enteritidis* PT4 or *Staph. aureus* and dried for 3 h as described in section 2.8.2. Inoculated test surfaces recieved no further treatment (control), or were washed by complete immersion in 100 ml water or detergent solution. The washing solution was contained within large petri dishes (14 cm diameter) which were placed on the platform of an orbital shaking incubator (Innova 4000, New Brunswick Scientific, NJ, USA). The platform was gyrated at 90 rpm and 25 °C for 2 min. The detergents used were household dish detergent (2 % v/v solution of Fairy Liquid, Proctor and Gamble), industrial detergent (2 % v/v Liquid Multiclean SU 125, Unilever Industrial) or industrial sanitiser (1 % v/v Quatdet SU321, Unilever Industrial). Where detergent treatments were used, the treatment was followed by a 100 ml water rinse (5 sec).

Washed and unwashed (control) surfaces were overlaid with nutrient agar or gelatin agar and incubated, for up to 48 h, as described in Section 2.8.3. The number of viable cells which had formed colonies, was then determined by reaction with NBT, also as described in Section 2.8.3. The number of organisms present in wash fluids was also determined.

Wash fluids were plated directly on duplicate agar plates (10 x 20 µl drops per plate) and additionally 1 ml samples were inoculated into BHI broth (10 ml).

2.10. Killing bacteria on food and food contact surfaces.

2.10.1. Cell killing experiments using cells in suspension.

Overnight broth cultures of *L. monocytogenes*, *P. fluorescens*, *S. enteritidis*, *E. coli* and *Staph. aureus* were diluted 1/25 in fresh BHI and grown to the early stationary phase (approximately 4 h incubation), as described in Section 2.8.1. Stationary phase cultures were diluted 1/100 into test media, which were: trisodium phosphate (TSP), TSP plus sodium chloride (NaCl), TSP plus heat-inactivated newborn calf serum (up to 50 % v/v; Gibco) and TSP plus NaCl and serum; NaCl was used at a concentration of 0.8 M, concentrations of TSP ranged from 0.001 M to 0.15 M and are detailed in the results section (Section 4.2.). After incubation in test media for 10 min at 4 °C or 37 °C, cell suspensions were further diluted (1/100) in distilled water or distilled water containing 20 µg ml^{-1} lysozyme (Sigma, L-6876). After incubation for a further 30 min, cells were diluted as appropriate in deionised water and plated on nutrient agar. Plates were incubated for up to 48 h at 37 °C or 30 °C (*P. fluorescens* only).

Experiments using *Campylobacter jejuni* were conducted in a similar manner except that the organism was initially grown in enriched brain heart infusion broth in an atmosphere with elevated CO_2 and reduced O_2 (Section 2.3). Viable counts of *C. jejuni* were determined on blood agar plates (Section 2.3) also incubated in an atmosphere with elevated CO_2 and reduced O_2 (Section 2.3).

2.10.2. Cell killing experiments using cells adhered to food surfaces.

2.10.2.1. Inoculation of food samples.

Food samples were immersed in early stationary phase cultures of test bacteria for 30 min at ambient temperature and then dried for 3 min in a stream of cold air, or alternatively, the surfaces of the samples were inoculated with 20 µl of the test culture and left to air dry for 30 min. The bacterial cultures were prepared as described in Section 2.3. The experimental food samples used were: 1g fresh chicken skin samples (removed from wing or thigh portions and purchased from a local supermarket); medium sized (approximately 0.5 g) fresh-frozen prawns; and circular sections (diameter 3 cm) of lettuce leaves.

2.10.2.2. Treatment of inoculated foods and detection of survivors following stomaching of samples.

Inoculated food samples were transferred to 10 ml TSP solution, 10 ml TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water, as appropriate. After incubation on a rotary shaker (100 rpm, Gallenkamp) for 10 min at ambient temperature (20-25 °C), or 4 °C or 37 °C, the samples were shaken to remove excess fluid and transferred to 10 ml distilled water or distilled water containing 100 µg ml^{-1} lysozyme (Sigma; L-6876). In some experiments, nisin (3.33 x 10^{-5} M, Sigma) was added to the final treatment solution. After incubation for 30 min, samples were stomached individually for 2 min with 50 ml distilled water in a stomacher (Colworth 80, Seward Medical, UK). Serial dilutions of stomached samples, and where indicated, treatment fluids, were plated on nutrient agar or blood agar (*C. jejuni* only). Plates were incubated as described previously.

2.10.3. Detection of survivors by an *in situ* method.

The experimental food surfaces used in these experiments were 1 g samples of irradiated chicken skin. These were aseptically removed from frozen irradiated chicken thighs kindly provided by Dr. Alexandra Carneiro de Melo. Isotron, Swindon, UK kindly carried out the irradiation.

Chicken skin samples were inoculated with *E. coli* as described in Section 2.10.2. They were subjected to various treatments with TSP, NaCl and lysozyme as described in Section 2.10.2 and overlaid with nutrient agar (Section 2.8.3).

2.10.4. Killing of cells adhered to food contact surfaces.

Surfaces (ceramic, stainless steel type 316; Section 2.7) were inoculated with *S. enteritidis* PT4 suspended in 10 % inactivated newborn calf serum and dried for 3 h as described in Section 2.9.1. Inoculated test surfaces received no further treatment (control), or were washed by complete immersion in 100 ml water or other treatment solution(s). The washing solutions were contained within large petri dishes which were shaken using an orbital shaking incubator at 25 °C for 2 min, as described in Section 2.9. The treatment solutions were 0.005 M TSP, TSP plus 0.8 M NaCl or 0.8 M NaCl and lysozyme (100 μg ml^{-1}) in deionised water. Viable colonies on washed surfaces were determined by overlaying with nutrient agar and, after up to 48 h incubation, reaction with NBT (Section 2.8.3). The number of organisms present in wash fluids was also determined. Wash fluids were plated directly on duplicate agar plates (10 x 20 μl drops per plate).

2.11. Calculations.

2.11.1. Viable counts.

Viable counts were determined by the *in situ* NBT method (Section 2.8.3) or by a surface-drop plate method (Postgate, 1969). For the surface-drop plate method, a tenfold dilution series of the bacterial suspension was prepared in quarter strength Ringer's solution. Ten x 20 µl drops of appropriate dilutions were then plated out, in triplicate, on nutrient agar or blood agar (*C. jejuni* only). After incubation (Section 2.3), viable counts were determined from dilutions giving up to approximately 50 colonies per drop.

The 95% confidence limits of viable counts were determined using the formula:

$$95\% \text{ confidence limits} = (c \pm 1.96 \sqrt{c})\, d/\, v$$

where c was the total number of colonies counted, d the dilution factor and v the volume of the dilution plated out (Meynell and Meynell, 1970). The 95 % confidence limits of all viable counts were generally within ± 10 % and always within ± 15 % of the values presented, except where very low survival rates were observed and the data are given to only one significant figure.

All experiments were replicated. Unless otherwise stated, the data presented are the means of two or more replicate experiments.

SECTION III.

Results: Microbial colonisation and survival on surfaces.

3.1. Comparison of a novel *in situ* nitroblue tetrazolium method with a conventional swabbing technique for enumeration of viable bacteria on surfaces.

3.1.1. INTRODUCTION

A key factor in studies of microbial adhesion and survival on food preparation surfaces is the efficiency of the sampling techniques used to detect adhered organisms. In the most widely used sampling methods, organisms are removed by swabbing, rinsing or adhesion to agar (Patterson, 1971). In such methods, the efficiency of recovery of organisms is generally low and may vary for different surfaces (Eginton *et al.*, 1995), or for the same surface exposed to differing treatments or contaminated with different populations. The subsequent recovery of microorganisms, successfully detached from surfaces by swabbing, may also be as low as 10 %, primarily due to the tight adherence of the bacteria to the swab material (Niskanen and Pohja, 1977).

In an attempt to avoid the uncertainty of data generated by sampling microbial populations on surfaces, an *in situ* method for enumerating viable bacteria on test surfaces was developed within our group. In the method, experimentally-contaminated surfaces (contained within large glass petri dishes) were overlaid with agar and after incubation, colonies were visualised by a further period of incubation with the redox dye, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride (nitroblue tetrazolium or NBT). Tetrazolium salts are widely used indicators of respiratory activity in microbial cells (Marshall *et al.*, 1995) and have been used in conjunction with bright field microscopy to assess the viability of individual cells on surfaces (Thom *et al.*, 1993). In the present application, NBT was reduced from a pale-yellow, water-soluble salt to a brightly coloured (blue/purple) insoluble formazan which stained growing colonies at the agar-test surface interface.

In the experiments described in this section (Section 3.1), the efficiency of the *in situ* method was compared to a conventional swabbing technique. The swabbing method used was based on that originally described by Gilbert and Maurer (1968). The method employs two cotton wool swabs. The first is moistened before application to the surface and the second swab is applied dry to remove any residual fluid. The solution used to

moisten the swab and resuspend organisms from swabs was SRM (swab resuscitation medium), as formulated by Dr. T. Donovan (cited by Humphrey *et al.* 1995). This medium was used in an extensive and recent Department of Health survey of bacteria on surfaces in catering establishments (Personal communication, 1995). The medium contains NaCl (to provide a suitable osmotic strength), peptone, sodium thiosulphate to neutralise any residual chlorine in samples, and Tween 80 which acts as a surfactant and neutraliser of phenolic disinfectants (Sveum *et al.*, 1992). Bacteria adhered to cotton wool were removed by vortexing for approximately 1 min. Longer periods of vortexing do not improve the efficiency of bacterial recovery (Chamberlain *et al.*, 1997).

3.1.2. RESULTS

3.1.2.1. Comparison of the *in situ* NBT method with a conventional swabbing technique for detection of adhered organisms.

The procedure adopted for detecting bacterial colonies at agar-test surface interfaces was as described in Section 2.8.3. Nitroblue tetrazolium is toxic to cells at the concentration (1 gL^{-1}) required to produce deeply stained colonies (Barnes *et al.*, 1996). Thus, bacteria on test surfaces were overlaid with agar and NBT was added to plates after incubation and colony formation. This approach enabled the visualisation of otherwise non-visible colonies at the test surface-agar interface. Fig. 3.1.1. shows the appearance of a representative test surface (ceramic), inoculated with a suspension of *Staph. aureus* and subsequently allowed to dry before overlaying with agar, incubating and developing with NBT. It is seen that small colonies are readily detectable on the opaque ceramic surface enabling quantitative enumeration of colony forming units. The technique was equally suitable for the detection of *E. coli* and *Staph. aureus* on glass, plastic and stainless steel surfaces.

The efficiency of the *in situ* method in detecting viable *E. coli* cells dried on surfaces was compared to that of a swabbing method. In initial experiments, *E. coli* was suspended in deionised water or 1/4 strength Ringer solution, prior to application to test surfaces. In these experiments, cell survival was low and apparently dependent upon inoculum size (see Section 3.2.3.1). However, higher recoveries not dependent upon inoculum size were observed when cells were suspended in BHI. The results given in Table 3.1 using BHI suspended cells, show that swabbing detected between only 15 and 22 % of viable *E. coli* cells detectable by the *in situ* method. In addition, the *in situ* NBT method was more sensitive than the swabbing method. When *E. coli* cultures (approximately $5x10^9$ cfu ml^{-1}) were diluted by a factor of 10^{-5} and applied (20 µl drops) to test surfaces, the mean number of colonies per drop in the *in situ* detection method was 90. In contrast, to

obtain an equivalent number of colonies per plate using the swabbing method, the 10^{-3} dilution of the *E. coli* culture had to be applied to the test surfaces.

The number of viable bacteria remaining on test surfaces after swabbing was determined by overlaying plates with agar and detecting colony formation, after incubation, using NBT. The results (Table 3.1.1) suggest that, on the basis of the data obtained using the *in situ* method, more than 95 % of *E. coli* cells were removed by the swabbing procedure. Thus, the poor recovery obtained using the swabbing method may indicate that removed cells may adhere tightly to swabs. However, it is also possible that the rehydration and dehydration procedures associated with swabbing reduced the viability of attached cell populations which remained attached to the test surfaces.

TABLE 3.1.1. Comparison of the *in situ* NBT method with a conventional swabbing technique for detection of adhered organisms.

Ten-fold serial dilutions of *E. coli* suspended in BHI were dried on glass, plastic and stainless steel surfaces for 3 h. The undiluted cell suspensions contained 4.6 x 10^9, 4.3 x 10^9 and 4.2 x 10^9 cfu ml^{-1}, respectively. Bacteria in dried films were either removed by swabbing and plated out, or detected by overlaying with agar and visualising with NBT after overnight incubation. The number of viable organisms remaining on test surfaces after swabbing was also determined by the NBT method.

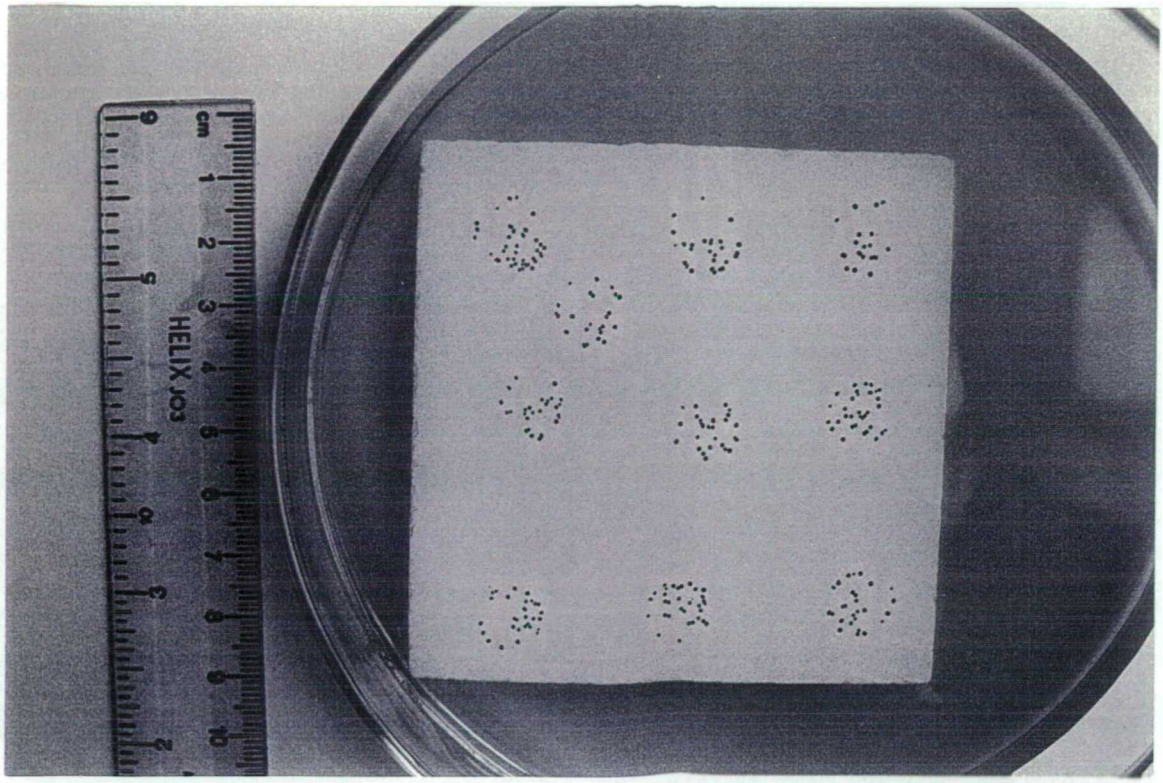
	Bacteria recovered from different surfaces expressed as cfu per ml of undiluted suspension		
	glass	**stainless steel**	**plastic**
Recovery by swab method*	4.90 x 10^8	1.34 x 10^8	3.80 x 10^8
Bacteria remaining on plates after swabbing**	1.40 x 10^8	1.25 x 10^7	3.00 x 10^7
Recovery by *in situ* method**	2.9 x 10^9	9.08 x 10^8	1.70 x 10^9
% Recovery of swabbing method compared to *in situ* method	17	15	22

*Determined using surfaces inoculated with 10^{-5} dilution of cells.

**Determined using surfaces inoculated with 10^{-3} dilution of cells.

Fig. 3.1.1. The use of NBT to visualise *Staph. aureus* colonies on a Ceramic **surface contained within a 14 cm diameter glass petri dish.**

Drops (20 µl) of a suspension of *Staph. aureus* in 10% serum were applied to the surface (9x9 cm^2) and, after drying for 3 h, at 37 °C, the surface was overlaid with agar. Plates were then incubated for 14 h at 37 °C to allow colony growth and overnight at room temperature, with NBT, to allow formazan (purple) production.



3.1.3. DISCUSSION

The approach used here for the detection of viable bacteria attached to test surfaces proved highly satisfactory. After overlaying surfaces with agar, and incubating to allow microcolony formation, approximately 6 h further incubation with NBT at room temperature was required to obtain deeply stained (purple) colonies of either *E. coli* or *Staph. aureus*. In subsequent experiments described in this thesis, the method was also successfully used to detect a range of additional Gram +ve (*L. monocytogenes*) and Gram -ve (*P. fluorescens*, *S. enteritidis*) organisms. Thus, it would appear likely that the technique would be suitable for use with all organisms capable of oxidising extracellular substrates. This would include the majority of foodborne pathogenic and spoilage bacteria. The technique would also appear suitable for a wide range of test surfaces, and in addition to glass, plastic and stainless steel, was successfully used with poultry skin (Section 4, this thesis) and wood (Barnes, 1998). Importantly, the presence of viable organisms within dried films of opaque material, for example serum (see Section 3.2), serum proteins and milk (Barnes, 1998), could also be detected.

In previous quantitative studies of survival on surfaces, a major factor affecting the interpretation of results was the efficiency with which attached organisms could be removed by swabbing or contact with solid media (Patterson, 1971). The *in situ* NBT method, as used in the present study, overcomes this difficulty and provides a standard against which other methods can be compared. The numbers of bacteria detected by the *in situ* NBT method was 5 to 6 fold greater than by swabbing. In swabbing methods, counts will be reduced as a consequence of the failure to completely remove adhered organisms from the test surface or from swabs used for sampling. The number of bacteria detected on test surfaces after swabbing was relatively small, approximately 10 % of those recovered by the *in situ* method. This suggests that swabbing is efficient in removing organisms from the test surfaces. However, it is possible that the stresses to which bacteria are subjected during swabbing may be greater than in the *in situ* method. The swabbing technique employed was based on that recommended by Gilbert and

Maurer (1968) and two swabs were used per surface. The first was applied moist and the second dry. It is possible that in such procedures some bacteria are subjected to partial rehydration/dehydration steps, leading to a reduction in the numbers of viable bacteria recovered and/or remaining on the test surface.

A further advantage of the *in situ* test method is its sensitivity. To obtain statistically reliable data (95 % confidence limits $< \pm$ 15 %) counts of more than approximately 200 colonies are required (Meynell and Meynell, 1970). In the *in situ* method, using for example *E. coli*, such numbers were detectable in dried films from 10 replicate 20 µl drops of suspension, initially containing only approximately 10^4 cfu ml^{-1} (Table 3.1.1). In contrast, where equivalent films were sampled by swabbing, adequate numbers of surviving bacteria were only detected when the suspensions used to inoculate surfaces contained more than 10^6 cfu ml^{-1}. The differences in apparent sensitivities of the methods are partly due to the higher recoveries of organisms in the *in situ* method. In the swabbing method, organisms are also diluted in swab suspension fluid.

A possible critisism of the *in situ* NBT technique is that it involves a small temperature stress on surface-attached organisms when they are overlaid with molten agar (45 °C). However, in later experiments (see Section 3.3), overlaying with gelatin agar (35 °C) did not significantly increase the recovery of bacteria, even after dried cells had been subjected to the additional stress of detergent treatment.

3.2. Application of the *in situ* NBT detection method to the evaluation of factors affecting the survival of microorganisms on surfaces.

3.2.1. INTRODUCTION

The ability of pathogenic and spoilage microorganisms to survive on surfaces within food processing and preparation environments may lead to contamination and cross-contamination of foods. The survival of microorganisms on such surfaces may be influenced by many factors.

In the food industry, the risks of contamination are considered to be greatly reduced when food contact surfaces are dry. However, non-spore forming bacteria may survive prolonged periods of desiccation (Chaturvedi and Maxcy, 1969). The survival of bacteria in films on solid surfaces is influenced by many extrinsic factors (Sveum *et al.*, 1992). These include: the nature of the medium in which cells are applied to the surface; the presence of moisture and the rate of drying; relative humidity (which also affects the rate of drying) and temperature.

The high susceptibility of bacteria to drying has been widely reported. For example, the count of *E. coli* suspended in broth medium and dried on glass coverslips, was reduced by up to 99 % after 24 h (Lemcke, 1959). In similar experiments, using *Pseudomonas aeruginosa* cells, survival was between 0 and 0.2 %, depending upon culture age (Skaliy and Eagon, 1972). More recently, Humphrey *et al.* (1995), noted the marked sensitivity of *Campylobacter* spp. to drying. The viability of organisms suspended in horse blood was initially unaffected when inoculated onto formica surfaces. However, when the suspension was allowed to dry, viable organisms could not be recovered.

The survival of dried bacteria on surfaces is generally stated to be greater at lower temperatures and relative humidity values (Sleesman and Leben, 1976). Thus, for example, Palumbo and Williams (1990) showed that the survival of *L. monocytogenes* in various simulated food products dried on surfaces was greater at 5 than at 25 °C and was higher at RH values of 59 % and below. However, McEldowney and Fletcher (1988) reported that the survival of *Pseudomonas* sp., *Acinetobacter calcoaceticus*,

Staphylococcus sp. and *Staph. aureus* on glass coverslips was similar at 0 %, 34 % and 75 % RH. Furtheremore, Helke and Wong (1994) showed that survival of *L. monocytogenes* and *S. typhimurium* on a variety of food contact surfaces at 6 °C, was higher at 75 % RH than at 30 % RH. This was attributed, in part, to a decreased rate of moisture loss at the lower RH value, which enhanced bacterial survival.

There are few studies which compare survival on different surfaces. Ak *et al.* (1994a, 1994b), showed that viable *S. typhimurium* cells were more readily recoverable from plastic than wooden surfaces; however, the role of the wooden surface in adsorbing the bacterial suspension applied was not clear. Mafu *et al.* (1990) showed that after short contact times (20 min, 1 h), the attachment of *L. monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces was similar. They also showed, using SEM, that for each of these test surfaces there were surface irregularities which might aid microbial attachment.

In addition to the various factors outlined above, bacterial survival on surfaces may be markedly affected by organism type, growth conditions and culture age (Sleesman and Leben, 1976; McEldowney and Fletcher, 1988). Skaliy and Eagon (1972) showed that for *P. aeruginosa*, optimal survival of cultures inoculated and dried onto a glass surface was obtained following 7 days incubation in growth medium. Similarly, Lemcke (1959) showed that for *E. coli*, optimal survival was at 18 h (early stationary phase) and that cells in the exponential phase of growth were particularly sensitive to drying.

The aim in this section of the thesis was to use the *in situ* NBT method to quantitatively and systematically determine: (i) the effect of drying time, temperature and RH on the survival of surface-adhered organisms; (ii) the relationship between the medium in which organisms were suspended and their subsequent survival when dried on surfaces; (iii) the influence of surface type upon bacterial survival; and (iv) the effect of surface abrasion on bacterial adhesion and survival. The organisms used included Gram +ve (*Staph. aureus* and *L. monocytogenes*) and Gram -ve (*P. fluorescens* and *S. enteritidis*) food spoilage

and pathogenic bacteria. *Escherichia coli* was also used as a 'model' organism. However, its survival may be similar to *E. coli* O157.

3.2.2. RESULTS

3.2.2.1. Survival of organisms suspended in deionised water and dried on surfaces: the effect of inoculum density.

Initially, it was considered that deionised water would be the most appropriate 'control' diluent for suspending cells in survival experiments. However, for *E. coli*, the proportion of cells recovered using deionised water varied and appeared dependent upon inoculum density. Thus, when tenfold serial diultions of *E. coli* suspensions were dried on glass or plastic surfaces, the number of viable bacteria detected by the *in situ* NBT method did not show the expected pattern, i.e. the patterns were not consistent with a tenfold dilution series. Instead, it was frequently observed that the number of colonies detected fell from an uncountable level at one dilution to zero, or a value close to zero, at the next dilution. This suggests that survival during the drying process is reduced at low population densities. Representative data is shown in Table 3.2.1. For suspensions containing 10^7 to 10^8 cfu ml^{-1}, the number of survivors was approximatelty 1 in 10^5 cells inoculated. In contrast, the survival of *Staph. aureus* suspended in deionised water and dried on surfaces appeared much less influenced by inoculum density (Table 3.2.1) and was approximately 0.3 % of the cells inoculated.

TABLE 3.2.1. The effect of population density on survival of *E. coli* and *Staph. aureus* suspended in water and dried on glass or plastic surfaces.

Various dilutions of suspended cells were inoculated onto the test surfaces and after drying in air (3 h) were overlaid with agar. Colonies were detected after incubation using NBT. The RH value was 40 %.

Organism and dilution of test suspension*	Surface	Mean number of colonies per 10 x 20 µl drops after drying	Surviving bacteria (cfu ml^{-1})
E. coli, expt. 1	glass		
10^{-1}		>2000**	>1 x 10^5
10^{-2}		310	1.6 x 10^5
10^{-3}		non detected	0
E. coli, expt. 2	plastic		
10^{-1}		>2000**	>1 x 10^5
10^{-2}		20	1.0 x 10^4
10^{-3}		non detected	0
Staph. aureus, expt. 1	glass		
1 x 10^{-3}		>2000**	>1 x 10^7
5 x 10^{-4}		1700	1.7 x 10^7
1 x 10^{-4}		110	5.5 x 10^6
5 x 10^{-5}		80	8.0 x 10^6
Staph. aureus, expt. 2	plastic		
5 x 10^{-4}		>2000**	>1 x 10^7
1 x 10^{-4}		520	2.6 x 10^7
5 x 10^{-5}		100	1.0 x 10^7

*Cells were grown in BHI and washed three times and resuspended in deionised water. Undiluted suspensions contained approximately 4 x 10^9 cfu ml^{-1} for *E. coli* and 3 x 10^9 cfu ml^{-1} for *Staph. aureus*.

** confluent growth.

3.2.2.2. The effect of drying time on the survival of bacteria inoculated onto test surfaces.

Cell suspensions of *Staph. aureus* were prepared in deionised water or BHI broth. Various dilutions of the suspensions were inoculated onto a plastic surface and after various drying times in air, the surface was overlaid with agar. After incubation, colonies at the test surface-agar interface were detected using NBT. Where inoculated cell suspensions on test surfaces had not completely dried before overlaying with agar, colonies were also distributed within the agar. The results, presented in Figure 3.2.1, show three phases of survival for cells suspended in deionised water. These were: (i) a period of little reduction in the number of viable organisms at drying times up to 1 h. At these times, the cells were present within liquid films; (ii) a period of rapid cell death, which coincided with the time at which films became completely dry (as assessed visually); and (iii) a period where viable cell numbers remained relatively constant in dried films. Surviving bacteria were still detectable at seven days (0.0012 % survival). In contrast, when *Staph. aureus* cells were suspended in BHI, cell numbers in liquid films increased slightly prior to drying, suggesting some growth, but there was little reduction in viability at the point of drying or during a further 42 h period.

Experiments similar to those with *Staph. aureus* were also conducted using *E. coli.* However the number of survivors was always lower for *E. coli* and, even in BHI, fell after 24 h of drying to less than 1 %. In both suspension media used (BHI, deionised water), the decline in viable cell number was most marked at around the time (1 h) at which the liquid film completely dried (Fig. 3.2.2). For cells suspended in deionised water, the results obtained are likely to be affected by inoculum density (See Section 3.2.2.1). The apparent decline in viable cell number at the point of drying was more than a 4 Log_{10} reduction.

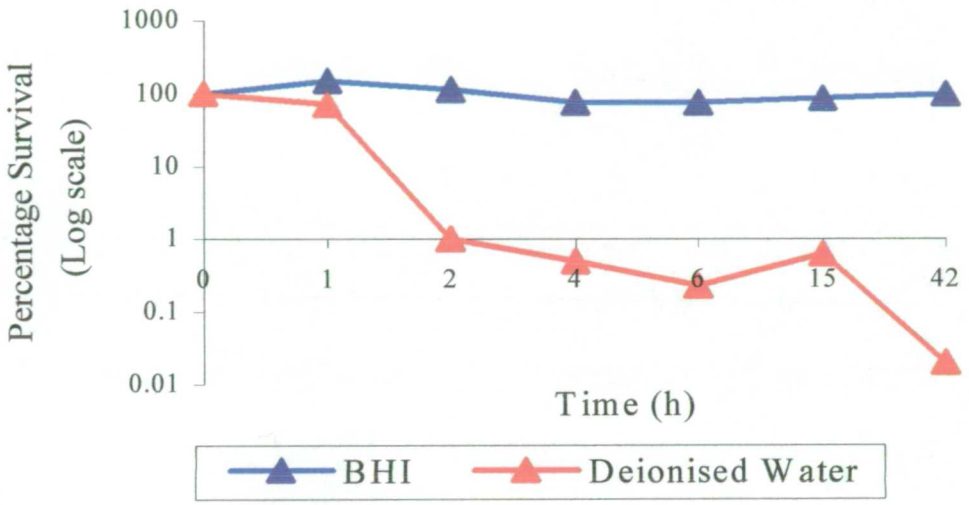


Fig. 3.2.1. The survival of *Staph. aureus* cells suspended in BHI or deionised water and dried for various times on plastic surfaces.

The moist films became dry after approximately 2 h. The RH value was 40 %.

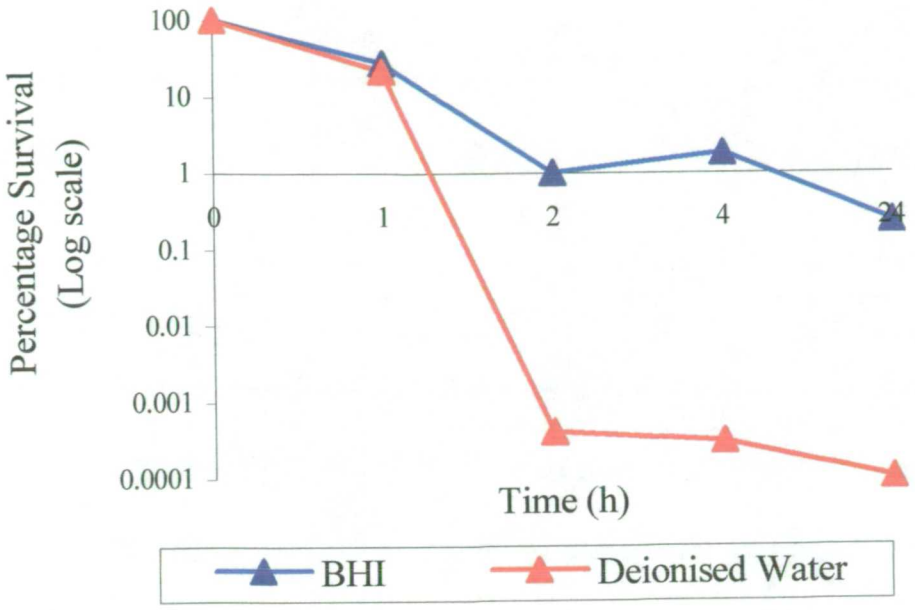


Fig. 3.2.2. The survival of *E. coli* cells suspended in BHI and deionised water and dried for various times on plastic surfaces.

The moist films became dry after approximately 2 h. The data for cells suspended in deionised water was based on an inoculum level of 2×10^9 cfu ml^{-1} suspension. The RH value was 40 %.

3.2.2.3. The effect of relative humidity on the survival of *E. coli* and *Staph. aureus* dried on surfaces.

Initially, the effect of RH on survival of *E. coli* cells inoculated onto plastic surfaces was determined. In these experiments, cells were suspended in 0.2% glucose since survival in deionised water was poor and the nutrient rich BHI medium (which gave relatively good survival) was both poorly defined and did not model suspension fluids likely to be present on food preparation surfaces. Relative humidity was controlled using a specially designed incubator (Stuart Scientific incubator SI60) with forced airflow over solutions of various water activity. Under these conditions, the time required for drops (20 µl) of microbial suspensions to visibly dry was less than 1 h at 30 % RH and approximately 1.5 h and 2.5 h at 50 and 80 % RH, respectively. The results for *E. coli* survival (Fig.3.2.3) were generally consistent with previous data (Fig. 3.2.2), in that initially survival was high but that large falls in viability took place at around the time of drying. In fully dried films (3-5 h drying time), the $\log_{10}$ reductions in viable count were 4, 6 and 6.5 at 30, 50 and 80 % RH, respectively.

The effect of RH on survival of *E. coli* and *Staph. aureus* was also determined for cells suspended in 10 % serum or 5 % NaCl, inoculated onto plastic or ceramic surfaces, and dried for a fixed period (3 h). The results for *E. coli* (Table 3.2.2.) show no consistent effect of RH (30-80 %) upon the numbers of organisms recovered, which was in all cases below 0.1 %. The survival of *Staph. aureus* was, as expected on the basis of earlier results, much higher than that of *E. coli*. For cells suspended in 10 % serum and inoculated onto both plastic and ceramic surfaces, survival was close to 100 %. For cells suspended in 5 % NaCl, survival varied between 4 and 34 %, though there was again no marked consistent effect of RH on survival. However, since the data in Table 3.2.2. was derived from a large number of experiments conducted on different days, it is possible that day to day variation in the preparation of cell suspensions may have masked any small influence of RH on survival. In all further experiments conducted, relative humidity was controlled at 38-40 % RH.

TABLE 3.2.2. The effect of relative humidity (RH) on the survival of *E. coli* and *Staph. aureus* on plastic and ceramic surfaces.

Cells were suspended in 10 % (v/v) serum or 5 % (w/v) NaCl, inoculated onto ceramic or plastic surfaces and allowed to dry for 3 h at 37 °C.

Suspending medium	Surface	% Survival			
		30% RH	40 % RH	50% RH	80% RH
E. coli					
10 % serum	plastic	0.0025	0.0075	0.0076	nd
	ceramic	0.0099	nd	0.0070	0.0087
5 % NaCl	plastic	0.014	0.08	0.055	nd
	ceramic	0.0070	nd	0.019	0.093
Staph. aureus					
10 % serum	plastic	100	98	95	93
	ceramic	76	98	100	84
5 % NaCl	plastic	16	9.0	8.4	34
	ceramic	4.5	11	3.6	16

nd, not determined.

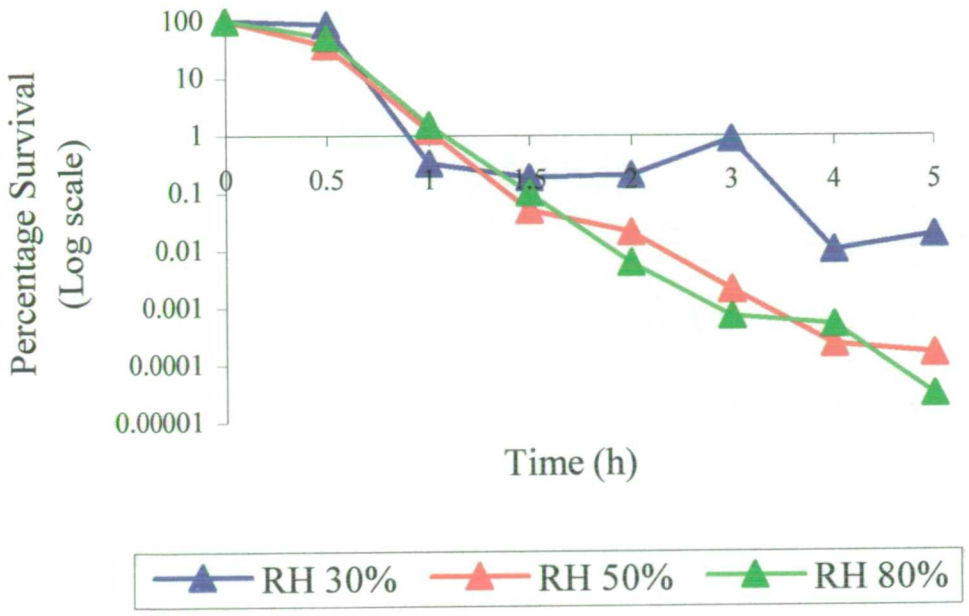


Fig. 3.2.3. The effect of relative humidity (RH) on the survival of *E.coli* suspended in 0.2 % glucose solution and inoculated onto plastic surfaces.

The number of viable cells were detected by the *in situ* NBT method after overlaying with nutrient agar and incubating for 24 h.

3.2.2.4. The effect of temperature on the survival of *E. coli* and *Staph. aureus* dried on surfaces.

The effect of temperature (10, 22 and 37 °C) on the survival of *E. coli* and *Staph. aureus*, suspended in serum (10 % v/v) or NaCl (5 % v/v) and dried on plastic or ceramic surfaces, is shown in Table 3.2.3. In serum, *Staph. aureus* showed high survival levels (≥ 95 %) at all temperatures. However, in NaCl (5 %), where survival levels were lower, the number of organisms recovered increased as the temperature of drying was reduced. Similarly, for *E. coli*, the highest survival levels (0.1 %) were observed at 10 °C, though the poorest survival (0.01 %) was at 22 °C.

TABLE 3.2.3. The effect of temperature (°C) on the survival of *E. coli* and *Staph. aureus* on plastic and ceramic surfaces.

Cells were suspended in 10 % (v/v) serum or 5 % (w/v) NaCl, inoculated onto ceramic or plastic surfaces and allowed to dry for 3 h at 37 °C.

Suspending Medium	Surface	% Survival 10 °C	22 °C	37 °C
E. coli				
10 % serum	plastic	0.10	0.011	0.076
5 % NaCl	plastic	0.17	0.015	0.055
Staph. aureus				
10 % serum	plastic	100	95	95
	ceramic	100	100	100
5 % NaCl	plastic	27	19	8.4
	ceramic	47	6.7	4.0

3.2.2.5. The effect of the suspending medium on the ability of *E. coli* and *Staph. aureus* cells to survive drying.

The results already obtained (Sections 3.2.2.2 - 3.2.2.4) show that the nature of the suspending fluid in which cells are applied to surfaces markedly affects their survival upon drying. The effect of NaCl, serum and sucrose concentration (0.1 to 10 %), in cell suspension fluids, was also determined. For *Staph. aureus* cells, suspended in deionised water and dried on glass and plastic surfaces, survival after 3 h was between 0.25 and 0.5 % (see Table 3.2.4; page 98). These values were significantly increased using only 0.1 % concentrations of the test suspension fluids. Survival increased further with increasing concentration of the suspension fluids and maximum survival values were >80 % with serum and sucrose and 8-12 % with NaCl. However, survival was also influenced by surface type (see Figs. 3.2.4. - 3.2.6 and Section 3.2.2.6). In 1/4 strength Ringer solution (sodium chloride 0.23 %, potassium chloride 0.01 %, calcium chloride 0.01 % and sodium bicarbonate 0.005 %), survival of *Staph. aureus* on glass and plastic surfaces (approximately 10 %) was similar to the maximum obtained using NaCl.

Data obtained for *E. coli* (Fig. 3.2.7), show that low concentrations of sucrose (0.1% w/v) and NaCl (1 % w/v) in the cell suspension medium also promoted survival following drying on a plastic surface. However, consistent with previous data, maximum survival rates (at 5 % sucrose and 10 % NaCl) were less than approximately 0.1 %. In 1/4 strength Ringer solution, survival of *E. coli* (0.0012 %) was similar to that for 1 % w/v NaCl.

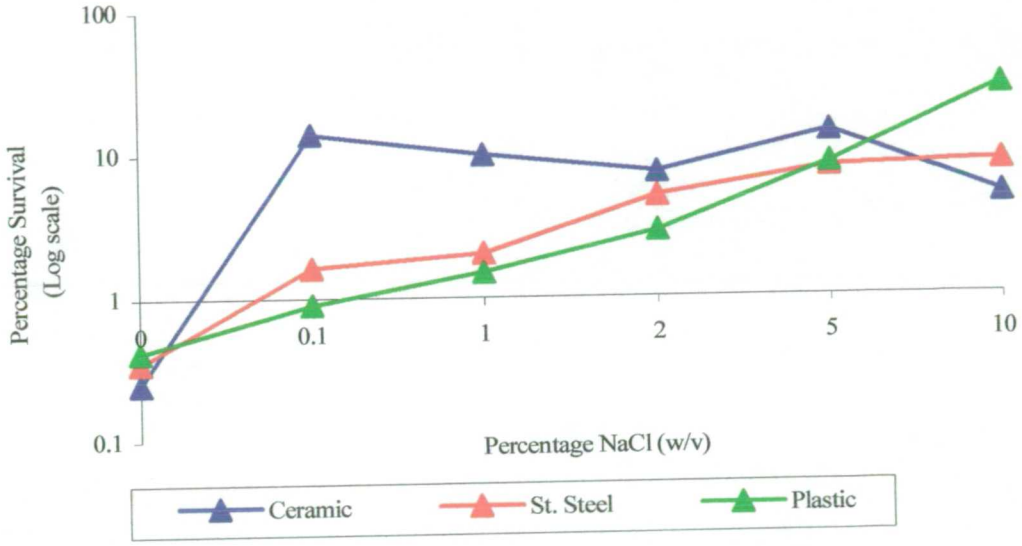


Fig. 3.2.4. The survival of *Staph. aureus* suspended in various concentrations of NaCl and dried on ceramic, plastic and stainless steel surfaces.

The numbers of viable cells were detected by the *in situ* NBT method after overlaying with nutrient agar and incubating for 24 h.

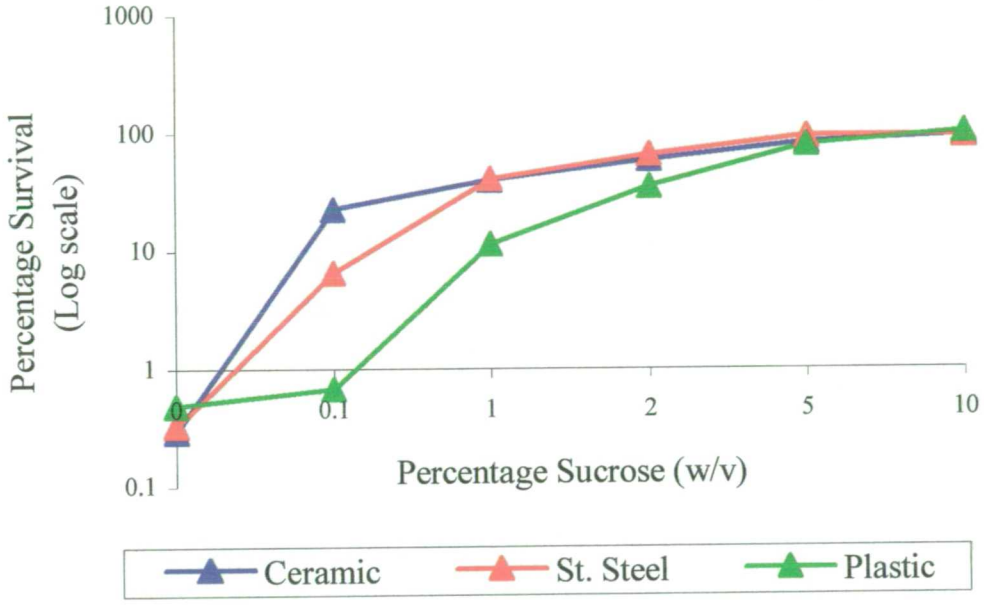


Fig. 3.2.5. The survival of *Staph. aureus* suspended in various concentrations of sucrose and dried on ceramic, plastic and stainless steel surfaces.

The numbers of viable cells were detected by the *in situ* NBT method after overlaying with nutrient agar and incubating for 24 h.

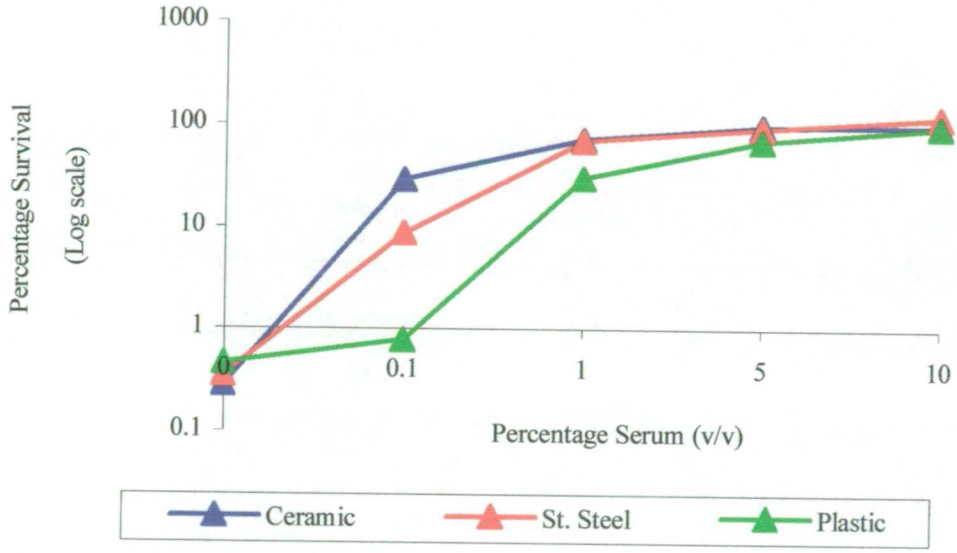


Fig. 3.2.6. The survival of *Staph. aureus* suspended in various concentrations of serum and dried on ceramic, plastic and stainless steel surfaces.

The numbers of viable cells were detected by the *in situ* NBT method, after overlaying with nutrient agar and incubating for 24 h.

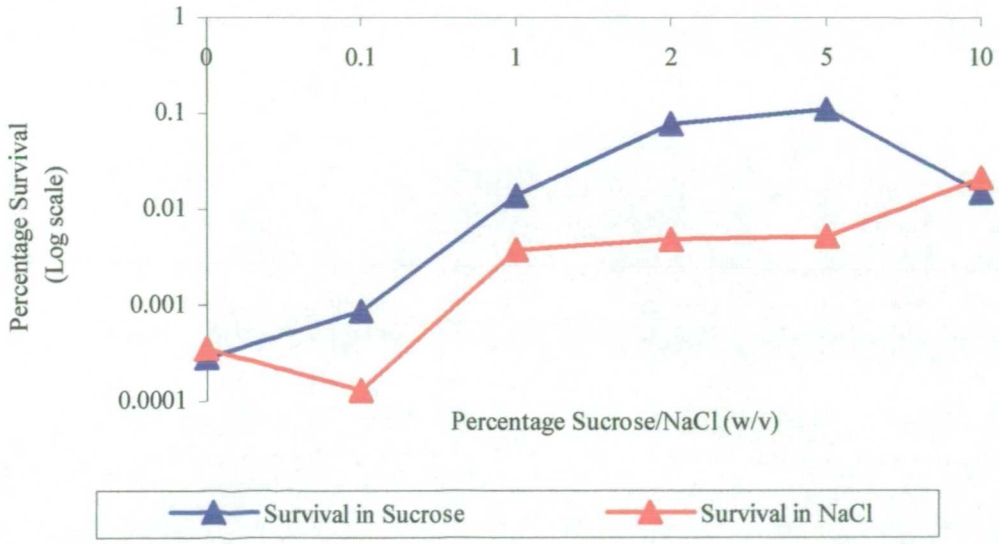


Fig. 3.2.7. The survival of *E. coli* suspended in various concentrations of sucrose and NaCl and dried on plastic surfaces.

The numbers of viable cells were detected by the *in situ* NBT method after overlaying with nutrient agar and incubating for 24 h.

TABLE 3.2.4. The survival of *E. coli* and *Staph. aureus* suspended in deionised water or Ringer solution and dried on plastic or glass surfaces for 3 h.

Surface	Suspension medium	% Survival
E. coli		
Plastic	deionised water	0.00033
	Ringer solution	0.00035
Glass	deionised water	0.0004
	Ringer solution	0.005
Staph. aureus		
Plastic	deionised water	0.38
	Ringer solution	0.84
Glass	deionised water	0.44
	Ringer solution	10

3.2.2.6. The effect of surface type on the ability of *E. coli* and *Staph. aureus* cells to survive the drying process.

Bacterial survival on surfaces impervious to water, i.e. ceramic, glass, plastic and stainless steel, were compared. Survival was generally little affected by the nature of the surface, as illustrated in the various experiments described above, which investigated the effects on survival of RH (Table 3.2.2), temperature (Table 3.2.3) and differences in the nature and concentration of cell suspension fluids (Figs. 3.2.4 - 3.2.6; Table 3.2.4). However, survival was frequently higher on ceramic, and plastic surfaces gave the poorest survival (see e.g. Table 3.2.7, page 99 and Figs. 3.2.4 - 3.2.6, particularly at low concentrations of suspending fluids). It was noted that this apparently reflected the mean drying times of drops (20 µl) applied to the surface, which was up to twice as long on plastic (90 min at 40% RH) than ceramic (30-40 min). On glass and stainless steel surfaces, drying times were approximately 60 min at 40 % RH. Consistent with these observations, it was also noted that the contact area of drops applied to ceramic tile was visibly greater than for the plastic surface, where drops appeared almost hemispherical. Thus, the observed effects of surface type on survival may be related to the rate of drying. This is discussed further in Section 3.3.

The effect of abrading surfaces, to simulate 'in use' wear, on the survival of bacteria applied to ceramic and stainless steel surfaces was determined. It was considered possible that bacterial entrapment and survival might be enhanced for organisms lodged in surface fissures of abraded or worn surfaces as proposed by Mafu *et al.* (1990). The abraded test surfaces used were polished stainless steel (commercially abraded, type 304 240 Si), which was compared to a smooth matt finish stainless steel (type 304 2B), and abraded ceramic tiles. These tiles were commercially abraded according to an industry protocol (see Section 2.7) and were compared to untreated tiles from the same source.

The abraded and unabraded test surfaces were inoculated with *Staph. aureus*, suspended either in serum (10% v/v) or NaCl (5 % v/v), and survival was determined after drying in air for 3 h. The results (Tables 3.2.5 and 3.2.6) show little effect of abrasion on survival.

TABLE 3.2.5. The survival of *Staph. aureus* on commercially polished (Type 304 240Si) and unpolished (304 2B) stainless steel.

Cells (approximately 3.0 x 10^9 cfu ml^{-1}) were suspended in either 5 % (w/v) NaCl or 10 % (v/v) serum. Ten x 20 µl drops, of a tenfold dilution series in the same diluents, were dried in triplicate, on the surface of stainless steel and abraded stainless steel for 3 h. The number of surviving cfu detected by the *in situ* NBT method was determined. The data shown is for a number of individual experiments conducted on different days.

Suspending medium	Mean cfu per 20 µl drop*	
	Type 304 2B Steel	Type 304 240 Si Steel
5% (w/v) NaCl	5.7	7.1
5% (w/v) NaCl	1.9	1.4
5% (w/v) NaCl	11	12
10 % (v/v) serum	81	80
10 % (v/v) serum	41	46

* 10^{-5} dilution of cell suspension for NaCl, 10^{-6} dilution of cell suspension for serum.

TABLE 3.2.6. The survival of *Staph. aureus* on commercially abraded and smooth finish ceramic wall tiles.

Cells (approximately 1.0×10^9 cfu ml^{-1}) were suspended in either 5 % (w/v) NaCl or 10 % (v/v) serum. Ten x 20 µl drops of a tenfold dilution series in the same diluents, were dried in duplicate, on the surface of ceramic tile and abraded abraded ceramic tile for 3 h. The number of surviving cfu detected by the *in situ* NBT method was determined. The data shown is for a number of individual experiments conducted on different days.

Suspending medium	Mean cfu per 20 µl drop	
	Smooth finish tile	Abraded finish tile
5% (w/v) NaCl	16	17
5% (w/v) NaCl	21	30
10 % (v/v) serum	11	6.7
10 % (v/v) serum	30	8.4

* 10^{-5} dilution of cell suspension for NaCl, 10^{-6} dilution of cell suspension for serum.

3.2.2.7. The survival of *L. monocytogenes*, *P. fluorescens* and *S. enteritidis* on ceramic and stainless steel surfaces.

The survival of *L. monocytogenes*, *P. fluorescens* and *S. enteritidis*, suspended in deionised water, NaCl (5 % v/v), and serum (10 % v/v), and dried on the surface of ceramic tile and stainless steel, was determined (Table 3.2.7). As observed for *Staph. aureus* and *E. coli* (Figs. 3.2.4., 3.2.5. and 3.2.6.), survival was generally greater in serum than in NaCl and the poorest survival was obtained for cells suspended in deionised water. Survival of *L. monocytogenes* was markedly better than that of the two Gram -ve bacteria and almost as high as that of *Staph. aureus* (see Tables 3.2.2. and 3.2.3.). Survival rates of *S. enteritidis* were generally close to those for *E. coli.* However, low survivals *of P. fluorescens* were obtained, even for cells suspended in serum. There was little difference between survival on ceramic and stainless steel surfaces except in experiments using cells suspended in deionised water. In these experiments, as previously observed for *E. coli* and *Staph. aureus* (Section 3.2.2.5), there was a high variability in survival in experiments conducted on different days. In the representative data shown in Table 3.2.5, survival was higher on ceramic for *L. monocytogenes* and on stainless steel for *P. fluorescens* and *S. enteritidis*, however these trends were not consistent.

TABLE 3.2.7. The survival of *L. monocytogenes*, *P. fluorescens* and *S. enteritidis* on ceramic and stainless steel surfaces.

Cells were suspended in either deionised water, 5 % (w/v) NaCl or 10 % (v/v) serum and dried on the surface of ceramic or stainless steel (type 316) for 3 h.

Organism	Surface	% Survival		
		Water	NaCl	Serum
L. monocytogenes	Ceramic	0.38	0.90	32.0
	Stainless steel	0.020	0.50	29.0
P. fluorescens	Ceramic	<0.00001*	0.0010	0.010
	Stainless steel	0.0010	0.0001	0.013
S. enteritidis	Ceramic	0.0041	0.3	1.12
	Stainless steel	0.020	0.62	0.55

*no survivors detected.

3.2.3. DISCUSSION

The *in situ* NBT method used in this study appeared eminently appropriate for the quantitative elucidation of factors affecting the survival of microorgansms on surfaces. Its use avoided the uncertainty of sampling efficiency associated with swabbing and agar contact methods. The sensitivity of the method also allowed the detection of low numbers of viable bacteria, as low as a single organism on a 9 x 9 cm^2 test surface.

Initially, it was thought that deionised water would be an appropriate fluid in which to suspend cells prior to inoculation of test surfaces. However, there was a large and variable reduction in viability when *E. coli* and *Staph. aureus* cells were suspended in deionised water and dried on test surfaces. In addition, survival was higher where high cell densities were used. In contrast, when cells were suspended in fluids containing various organic and inorganic materials, survival was independent of inoculum density and much higher levels of survival were obtained. It was also shown that when cells were dried on surfaces, the major loss of viability was associated with the period of drying rather than the times when cells were present in aqueous films or (after drying) in dried films. These findings provide an explanation for the low and variable recoveries obtained when cells were suspended in deionised water prior to drying on surfaces. As water drops evaporate, even in cell suspensions in deionised water, there will be a concentration of trace quantities of solutes. In addition, cells lysed during the drying process may also release organic and inorganic compounds. Thus, survival may well be influenced by population density and by minor differences in the carry-over of nutrients in the preparation of washed cell suspensions. Previous studies of survival of bacteria on food contact surfaces have generally used cells suspended in complex materials, e.g. tryptic soy broth (Krysinski *et al.*, 1992; Blackman and Frank, 1996). However, Palumbo and Williams (1990) did show that survival of *L. monocytogenes* in distilled water was consistently poorer than in Karo syrup. Also, Jawad *et al.* (1996), have recently reported that the survival of *Acinetobacter* sp. increased from 11 days, for cells dried in distilled water, to 60 days for cells dried in 7 % w/v bovine serum albumin.

In studies of the effect of exposure time on the survival of cells inoculated onto surfaces, investigators have generally failed to provide precise information as to the relationship between cell survival and the presence of water/extent of drying. For example, in studies using *L. monocytogenes* and *S. typhimurium* (Helke and Wong, 1994), rates of water loss were not determined though it was noted that "the appearance of a visibly dry surface (for cells suspended in phosphate buffered saline) coincided with the lowest recovery of microorganisms". The time required to produce a visibly dry surface in these experiments was several days. McEldowney and Fletcher (1988) used glass coverslips which had been incubated in culture media and washed with water to study the survival of various bacteria. Surviving bacteria on air-dried coverslips were estimated semi-quantitatively by direct transfer to agar. The results appeared to show that viable cell numbers oscillated periodically (over a period of 100 days) and the authors speculated that this may have been due to the release of nutrients from lysed cells and the presence of water within the microcolonies. Thus again any relationship between viable count and the time of drying of aqueous films is obscure. In earlier work, Lemcke (1959) was precise concerning the time required to dry *E. coli* films on glass coverslips, which under her conditions was 2 h. However, the number of surviving bacteria, removed by washing, was determined only for dried films. Humphrey *et al.* (1995) were able to conclude that drying was associated with a significant fall in viability of *C. jejuni*. In their experiments, the organism was inoculated onto formica in blood droplets and incubated under conditions in which drops were allowed (open containers) or were not allowed (closed containers) to dry. After 4 h, survivors were not detectable by swabbing in dried films but had declined only from a mean count of 82 to 68 cfu per drop in undried films. Precise reductions in count could not be given because of the design of experiments.

In the experiments conducted here, the drying time was carefully recorded and the data clearly show that the major reduction in viable count of inoculated cells was at the time when the films became visibly dry. Consistent with the observations of Humphrey *et al.* (1995) for *C. jejuni*, there was little reduction in the viability of cells prior to the point of

drying. After drying, the viability of both *E. coli* and *Staph. aureus* declined slowly. Thus, in comparing the effect of physical and other factors on survival, the viability of bacteria was generally determined after 3 h. At this time, bacterial suspensions were visibly dry in all the experiments conducted.

The extent of the reduction in viability at the time of drying (Figs 3.2.1., 3.2.2.) or after drying (Figs. 3.2.4., 3.2.5., 3.2.6. and Table 3.2.1., 3.2.4) was markedly dependent upon the nature of the fluid in which cells were suspended. Recoveries of more than 30 %, and for some organisms up to 100 %, were frequently observed where bacteria were suspended in BHI, serum or sucrose. Bacterial survival in NaCl was also enhanced compared to that in deionised water. Survival generally increased as the concentration of the suspending medium increased; however, even low concentrations (0.1%) of NaCl, sucrose or serum markedly increased survival (Figs. 3.2.4 to 3.2.6). This would be expected, since if the major reduction in viable count occurs at the time of drying, the effective concentration of solutes at this time would be greatly increased as a result of evaporation.

Various mechanisms may be important in the ability of solutes to enhance survival. Metal ions, particularly divalent ions, may stabilise cell surface structures including the lipopolysaccharide of Gram-ve bacteria (Hancock, 1984). The presence of metabolisable, energy-yielding substrates (e.g. glucose in serum, BHI or as a contaminant of commercial sucrose preparations) will enable cells to respond to changes in external osmotic pressure, by uptake of ions and other substances or the synthesis of solutes (Csonka, 1989). This may be particularly important as changes in osmotic pressure during drying are likely to be very high, since immediately prior to drying, external solutes will be substantially concentrated.

Food may be stored or exposed to food contact surfaces under varying conditions of temperature and relative humidity. Previous results suggest that dried cells survive better at lower temperatures. For example, Sleesman and Leben (1976) reported that the

survival of *Corynebacterium michiganense*, *Xanthomonas phaseoli*, *Pseudomonas glycinea* and *Erwinia carotovora* attached to glass beads, was consistently better over a 30 day period at 5 °C than at 20 °C. Similarly, McEldowney and Fletcher (1988) showed that the survival of *Pseudomonas* sp., *A. calcoaceticus*, a *Staphylococcus* sp. and *Staph. aureus* attached to dry glass coverslips was shortest at 25 °C and longest at 4 °C; and Palumbo and Williams (1990), reported higher survival of *L. monocytogenes*, dried in beef extract or distilled water for 40 days on glass coverslips, at 5 °C than at 25 °C.

The influence of temperature (10, 22 and 37 °C) on the survival of the test bacteria in this study was complex. In agreement with previous reports, the survival of *Staph. aureus* was promoted at the lower temperature (10 °C). However, although survival of *E. coli* was highest at the lowest temperature tested (10 °C), minimum survival was at 22 °C. Since cells have a reduced metabolic activity at lower temperatures temperature may affect this ability to adapt to the rapidly changing external osmotic pressure associated with drying. This effect may be more important for Gram -ve cells which are much more susceptible to osmotic stress (Strange and Cox, 1976). Thus, for such cells, there may be two major effects of temperature, one favouring survival at higher temperature and one at a lower temperature. However, whilst it might be possible to explain the poor survival of *E. coli* at 22 °C in this manner, other factors may also be involved.

RH would be predicted to affect the survival of cells inoculated onto surfaces as a consequence of altered rates of water evaporation, changes in osmotic pressure of the surrounding medium, and by affecting the state of dehydration of cells. It is well known that the length of survival of freeze-dried culture stocks, for example, is dependent upon the extent of water removal. However, in the present study, relative humidity (30 - 80 %) had no consistent effect upon survival of *E. coli* and *Staph. aureus*. In qualitative experiments, McEldowney and Fletcher (1988) similarly reported that RH (0 to 75 %) had variable effects on the length of survival of Gram +ve (a *Staphylococcus* sp. and *Staph. aureus*) and Gram -ve (*Pseudomonas* sp. and *A. calcoaceticus*) bacteria in dried

films. Thus, for example, at 25 °C, *Staph. aureus* survived for 8 days at 0 % RH and 25 days at 75 %; whereas, in contrast, the survival times of a coryneform bacteria were 46 and 5 days respectively. Also, for an unamed *Staphylococcus* sp., survival was optimal at 34 % RH. In other reports, Jawad *et al.* (1996) showed that clinically-important *Acinetobacter* spp. survived longer at higher RH (31 and 93 % RH compared to 10 %) and Helke and Wong (1994) similarly found higher survival of *L. monocytogenes* and *S. typhimurium* at 75 % RH than at 32.5 % RH, at both 6 and 25 °C. In contrast, Palumbo and Williams (1990) reported that, at 25 °C, *L. monocytogenes* survived longer at lower RH values (1 and 14 % RH as compared to 35, 59 and 75 % RH). Finally, Sleesman and Leben (1976) reported that survival of a range of environmental organisms (*Cor. michiganense, Er. carotovora, P. glycinea* and *X. phaseoli*) was better at 34 % RH than 0 or 75 % RH; poorest survival was observed at 75 % RH.

Thus, it appears that the effects of RH on survival are complex, perhaps being influenced by uncontrolled parameters in the experiments conducted, for example cell drying rates. It is also possible that survival data recorded are dependent upon the methodologies used to recover (and where appropriate, enumerate) cells, particularly the ability to remove adherent organisms from test surfaces in well-dried films. The methodology used in the experiments reported in this thesis was consistent and independent of this factor. However, time did not permit the systematic comparison of the method with the various other methods which have been used to study the effects of RH on survival. In addition, it was shown that RH had a relatively minor effect on the survival of both Gram +ve and Gram -ve cells, compared to the role of the suspending fluid in which cells were dried. It was concluded that, in the design of experiments, an RH value of 40 % would be appropriate. This value was within the ambient RH range within the laboratory (35 to 45 %) and would appear typical of the values which might be experienced in kitchens.

Studies of the influence of surface type on bacterial contamination, have mainly been concerned with effects upon bacterial adhesion. These studies show that surface type does influence adhesion but that other factors, particularly the type of organism, may be

more important. For example, Eginton *et al.* (1995) reported that the attachment of bacteria to a range of surfaces (with the exception of stainless steel and formica) was species dependent, with the attachment of *P. aeruginosa* being almost twice that of *Staphylococcus epidermidis.* Mafu *et al.* (1990) suggested that the attachment of *L. monocytogenes* to four surface types including stainless steel and glass, was affected by the intrinsic properties of the surface but were also influenced by other factors related to the type of organism, such as exopolysaccharide production. Similarly, Suárez *et al.* (1992) reported that although there was no significant difference in the attachment of several strains of psychrotrophic bacteria (*Staphylococcus, Acinetobacter, Pseudomonas* and *Flavobacterium* spp. isolated from milk), to stainless steel and two types of rubber, there was significantly less attachment to glass.

In contrast to the information available concerning microbial attachment to surfaces, there is little in the literature regarding the effect of different surface types on the survival of adhered bacteria. In particular, there are few reports that specifically relate to the survival of bacteria dried on different types of food contact surfaces. Helke and Wong (1994) reported on the survival of *L. monocytogenes* and *S. typhimurium* inoculated onto the surface of stainless steel and buna-rubber. They examined bacterial survival over a 10 day period and showed that both organisms were able to survive on the test surfaces for prolonged periods under specific conditions of RH and temperature. However, bacterial survival did appear dependent on the surface type; both test organisms survived for longer time periods when dried on stainless steel. In contrast, Ak *et al.* (1994) investigated bacterial survival on wood and plastic surfaces, inoculated with suspensions of *E. coli, L. innocua* and *S. typhimurium,* and dried overnight. They found no significant difference in bacterial recovery with surface type; however, there were differences in recoveries amongst species.

In this thesis, survival of the test organisms appeared relatively independent of the test surfaces used (ceramic, glass, plastic and unpolished stainless steel). However, it was noted that survival was frequently higher on ceramic and lower on plastic surfaces. This

correlated with the mean drying times of drops (20 µl) applied to the surface, which was up to twice as long on plastic (90 min) than on ceramic (30-40 min). This presumably reflected the larger surface area of drops applied to ceramic tile, which would therefore evaporate more quickly. Rapid drying, particularly during the final stages of drying when cells are subjected to the greatest stress (highest rate of change in external osmotic pressure), may lead to enhanced recoveries. Eginton *et al.* (1995) assessed the wetting properties (hydrophobicity) of a selection of test surfaces by measuring the surface contact angles (air:water). Stainless steel (43 ± 10°) and glass (40 ± 6°) had similar contact angles. However, Pringle and Fletcher (1983) found that the contact angle of petri dish polystyrene was 90°. Such measurements confirm that polystyrene, having the greater contact angle, is more hydrophobic. Thus, as observed (see Section 3.1), drops formed on its surface will have relatively low surface area to volume ratios and will dry more slowly.

Since there was little variation in survival on surfaces of markedly different type, it was not expected that abrasion of stainless steel or ceramic surfaces (to represent used or worn surfaces) would lead to altered survival rates. This expectation was confirmed; survival rates on polished and standard mill finish stainless steel and new and abraded ceramic tiles were comparable (see Tables 3.2.5 and 3.2.6). Mafu *et al.* (1990) had proposed that irregularities on the surfaces of rubber, polypropylene, stainless steel and glass might enhance the adherence and survival of bacteria. However, they found no correlation between the presence of surface irregularities and the attachment of *L. monocytogenes.* Similarly, although Ak *et al.* (1994) were able to show that the recovery of *E. coli* inoculated onto new and abraded wooden and plastic chopping boards was dependent on the surface type (more bacteria were recovered from plastic), the condition of the surface did not appear important. However, this finding conflicts with a recent study by Welker *et al.* (1997). They reported the sporadic recovery of *E.*

coli from used wooden boards, whereas viable organisms were not detected on new wooden boards and both used and new plastic boards. Consequently, conclusions drawn from the few investigations that have been carried out to determine the effects of surface wear and abrasion on bacterial retention, are not in agreement. However, these variations may be in part attributable to the analytical approach used in the estimation of bacterial survival. The method used in the work reported in this thesis may be more reliable, as the data obtained are not affected by the low and variable efficiency of removing organisms from surfaces, before viable counts may be determined.

The type of organism used in this study appeared to have a profound effect on bacterial survival on surfaces, especially where cells were suspended in deionised water or low concentrations of solutes. Whilst only a relatively small number of bacteria were studied, it was apparent that the survival of the Gram +ve organisms was much greater than that of the Gram -ve organisms. This may reflect the importance of osmotic stresses during drying on viability, and the higher susceptibility of Gram -ve organisms to osmotic stress (Strange and Cox, 1976). However, under a selection of conditions (variable RH and temperature), McEldowney and Fletcher (1988) found no consistent difference in the survival times, in dried films, of *Staph. aureus* (Gram +ve), a *Pseudomonas* sp. and *A. calcoaceticus* (Gram -ve). Jawad *et al.* (1996) also investigated the survival times of Gram +ve and Gram -ve bacteria in dried films at different RH values. They divided the test organisms into two groups. *Staphylococcus aureus*, *Serratia marcescens* and *Acinetobacter spp.* were considered dessication resistant, as they survived for more than 11 days, whereas *E. coli* and *Enterococcus* spp., were classed as dessication sensitive as they survived for an average of only 5 days. Thus, in the study of Jawad *et al.* (1996), although marked differences in the survival times of adhered organisms was evident, Gram +ve organisms did not consistently survive longer than

Gram -ve organisms. It is possible that the fundamental differences between the results obtained in the work described in this thesis, and previous results reflect the experimental approach used, environmental conditions and physiological state of the organisms at the time of their application to the test surfaces. Importantly, in the present study, the *in situ* NBT method was used to determine the survival of organisms dried on surfaces. The methods used by other workers are subject to the fundamental criticism that they do not distinguish survival from ease of removal of bacteria from surfaces, since organisms were recovered from surfaces either by swabbing (e.g. Helke and Wong, 1994), rinsing (e.g. Palumbo and Williams, 1990; Ak *et al.* 1994a, 1994b; Pringle and Fletcher, 1983), or adhesion to agar (e.g. Ak *et al.* 1994a).

3.3. Application of the *in situ* detection method to the evaluation of decontamination procedures for food contact surfaces.

3.3.1. INTRODUCTION

Pathogenic microorganisms attached to food contact surfaces may be transferred to food and therefore constitute a potential health hazard. The transfer of spoilage microorganisms may also result in a shortened shelf-life of the foods involved. The regular cleaning of food contact surfaces is thus necessary to prevent contamination.

Generally, the aim of cleaning procedures is seen as the removal rather than the inactivation of microorganisms (Hood and Zottola, 1995). The desorption of microorganisms from food contact surfaces arises primarily as a consequence of sloughing off of cells from the surface due to shear forces during cleaning procedures. McEldowney and Fletcher (1988) demonstrated that bacterial desorption varied with surface composition (glass, tin plate, polypropylene, stainless steel and nylon) and the type of bacterial species (*Staph. aureus* and *A. calcoaceticus*) colonising the surface. Where cleaning treatments lead to bacterial desorption, there is a potential for subsequent contamination of other surfaces. Consequently, to minimise microbial dispersal, two step cleaning and sanitising procedures are recommended (Zottola and Sasahara, 1994) which lead to the removal of surface soil and subsequent inactivation of bacterial species present within the soil. Cleansing agents generally include components that 'wet' and penetrate the soil, thereby facilitating its removal. Disinfectants and sanitisers are more efficient after organic material has been removed from the surface, as this enables more effective contact with surface-attached microorganisms.

The effectiveness of surface decontamination has been widely studied because of the percieved importance of food contamination from contact surfaces (Zottola and Sasahara, 1994). The *in situ* NBT method was applied to decontamination studies because of the sensitivity of the method and the direct relationship between the numbers of colonies detected, after overlaying with agar, and the numbers of viable organisms present on the surface. One difficulty that was envisaged in applying the NBT method to decontamination studies was the possible effects of molten agar (at 45 °C) on the viability of cells stressed by treatments with detergents and sanitisers. Thus experiments were also conducted in which contaminated surfaces were overlaid with gelatin agar, which may be used molten at 35 °C. Gelatin agar solidifies at temperatures less than 30 °C, and in these experiments, the subsequent incubation temperature was 25 °C. The test organisms used were *Staph. aureus* and *S. enteritidis* and the test surfaces were ceramic tile and stainless steel. Cleaning regimens were based on the use of water, household (Fairy Liquid) and industrial (Multiclean) detergents and an industrial quaternary ammonium sanitiser (Quadet). The Fairy Liquid was used at 2% v/v and the industrial products at the concentrations recommended by the manufacturer.

3.3.2. RESULTS

3.3.2.1. Decontamination of surfaces.

Drops (20 µl) of *S. enteritidis* and *Staph. aureus* suspended in 10 % serum or 5 % NaCl, were dried on 9 x 9 cm^2 ceramic and stainless steel surfaces at initial population densities of 10^3 to 10^6 cfu per drop. The *in situ* NBT method was then used to assess the ease

with which surfaces could be decontaminated using a two minute wash with 100 ml of water, household dish-washing detergent (2 %), industrial detergent (2 %) or industrial sanitizer (1 %); where detergents or sanitizers were used, treatment was followed by a brief (5 sec, 100 ml) rinse in deionised water. The results (Table 3.3.1) show that for both test organisms dried in 10 % serum or 5 % NaCl, simply washing in water reduced the number of viable adhered organisms to approximately 0.1 - 1 % of pre-wash values, i.e. the reduction in count was about 2 to 3 $\log_{10}$ cycles. Washing in household detergent caused a greater decrease in adhered bacteria (up to 4 $\log_{10}$ cycles), which was more marked for cells that had been dried in serum than in NaCl. In all cases, the industrial detergent was more effective in decontaminating surfaces than household detergent and, with the industrial sanitizer, viable organisms were not detected. There were no consistent differences in results for the two surfaces.

The number of viable organisms present in wash fluids was also determined (Table 3.3.1). Following washing with deionised water, up to almost 20 % of the organisms which survived drying on the test surfaces, were recovered in the water used for washing. However, the numbers recovered were considerably greater where cells had been dried in serum (3 - 17 % of viable attached bacteria) than in NaCl (0.2 - 2.7 %). Also, for cells dried in serum, recovery of *S. enteritidis* (11 - 17 %) was higher than for *Staph. aureus* (3 - 10 %). For cells dried in NaCl, recoveries of the two organisms were similar (up to approximately 3 %).

Compared to washes with deionised water, the number of *Staph. aureus* recovered in wash fluid containing household detergent was reduced by up to more than tenfold.

However, the detergent appeared ineffective against *S. enteritidis* and recoveries were, in all cases, comparable to those in water. The industrial detergent and sanitiser were more effective against both organisms and viable organisms were not recovered in wash fluids. Consistent with these observations, in experiments on suspended cells (Appendix I), it was shown that a 2 min exposure to household detergent or industrial detergent (2 % v/v) reduced the number of viable *Staph. aureus* cells by 84 and 100 % respectively, whereas the corresponding kills for *S. enteritidis* were 10 % and 80 %. The sanitizer was completely effective against both organisms (10^6 cfu ml^{-1}) at the concentration recommended for use by the manufacturer.

TABLE 3.3.1. The decontamination of surfaces by washing with water, household dish-washing detergent, industrial detergent and industrial sanitizer.

Surfaces were inoculated with 20 µl drops of various dilutions of bacteria (10^3 - 10^6 cfu per drop) suspended in 10 % serum or 5 % NaCl and dried. The numbers of organisms recovered on washed surfaces and in wash fluids is expressed as a % of those detected on unwashed surfaces by the *in situ* NBT method.

Organism and suspending medium	Surface	Washing fluid	Organisms recovered (% of organisms recovered from unwashed surface)†	
			Washed surface	Wash fluid
Staph. aureus				
10 % serum	ceramic	water	1.8	10.2
10 % serum	ceramic	detergent (H)	0.048	ns
10 % serum	ceramic	detergent (I)	0.0038	ns
10 % serum	ceramic	sanitizer	ns	ns
5 % NaCl	ceramic	water	1.03	2.69
5 % NaCl	ceramic	detergent (H)	0.46	ns
10 % serum	stainless steel	water	0.48	4.1
10 % serum	stainless steel	detergent (H)	0.084	1.6
5 % NaCl	stainless steel	water	0.15	3.4
5 % NaCl	stainless steel	detergent (H)	0.15	0.84

Continued

Table 3.3.1. Continued.

S. enteritidis				
10 % serum	ceramic	water	0.18	16.7
10 % serum	ceramic	detergent (H)	0.024	18.9
10 % serum	ceramic	detergent (I)	ns	ns
10 % serum	ceramic	sanitizer	ns	ns
5 % NaCl	ceramic	water	0.18	0.21
5 % NaCl	ceramic	detergent (H)	0.11	0.07
10 % serum	stainless steel	water	0.093	11.4
10 % serum	stainless steel	detergent (H)	0.0087	16.0
5 % NaCl	stainless steel	water	1.52	0.20
5 % NaCl	stainless steel	detergent (H)	0.60	0.30

ns, no survivors detected.
H, household detergent.
I, industrial detergent.

† The numbers of organisms detected on unwashed surfaces were approximately: 85 % and 1 % of those inoculated, for *Staph. aureus* dried in serum and NaCl respectively; and ≤ 0.05 % for *S. enteritidis* dried in serum or NaCl.

3.3.2.2. The use of gelatin agar to overlay test surfaces.

In the *in situ* NBT method (Section 3.1), test surfaces were overlaid with nutrient agar at 45 °C. To determine whether the agar temperature reduced bacterial survival, the use of nutrient agar was compared to that of gelatin agar (overlaid at 35 °C). The results presented in Table 3.3.2. show that the number of viable *Staph. aureus* cells detected on unwashed, water-washed and detergent washed ceramic surfaces was similar for both agar types.

Table 3.3.2. The use of gelatin and nutrient agar overlays in the detection of *Staph. aureus* cells on a ceramic surface.

Ceramic surfaces were inoculated with 20 µl drops of bacteria suspended (10^3 - 10^6 cfu per drop) in 5 % NaCl and dried. The surfaces were then either left unwashed or were washed in deionised water or household detergent (household dish detergent; 2 % v/v solution), before overlaying with either nutrient or gelatin agar. The numbers of bacteria detected are expressed as a % of those originally applied to the surface.

Wash treatment and agar overlay	Organisms recovered (%)
Unwashed / Nutrient Agar	15.5
Unwashed/ Gelatin Agar	7.11
Water washed / Nutrient Agar	0.0245
Water washed / Gelatin Agar	0.0306
Detergent washed/ Nutrient Agar	0.0094
Detergent washed / Gelatin Agar	0.0121

3.3.3. DISCUSSION

It is acknowledged within the food industry that the most common cleaning medium is water (Sprenger, 1989), which has the ability to dissolve many residues and in turn forms a solution that can be easily washed away. However, water is not efficient in dissolving many of the residues that are commonly encountered in the context of food preparation (Bloomfield and Scott, 1997). Consequently, chemical agents such as detergents are required.

In previous studies, wiping and spraying (Stevens and Holah, 1993) or rinsing (Bloomfield *et al.*, 1994) artificially contaminated surfaces with water gave an approximately 1 $\log_{10}$ reduction in the numbers of attached bacteria, detectable by swabbing. Using the *in situ* NBT method, more than approximately 99 % of viable organisms adhered directly on ceramic and stainless steel surfaces were removed by a two minute wash with deionised water, even where the organisms had been applied to the surface in 10 % (v/v) serum. Interestingly, for both *Staph. aureus* and *S. enteritidis*, the total number of viable organisms recovered following washing (i.e. the count on the washed surface plus the counts for the wash fluid), was less than 20 % of the count on unwashed surfaces. Thus, most of the organisms which could be detected by overlaying with agar were killed by direct rehydration with water. This is consistent with the enhanced recovery of organisms on surfaces by the *in situ* NBT method developed here, compared to the much lower recovery using moistened swabs (section 3.1).

The number of bacteria detected in wash water was much greater where cells had been dried in serum than in NaCl. This may indicate that the presence of serum not only promotes cell survival during dehydration (see Section 3.2), but also protects cells during rehydration with deionised water. The number of bacteria detected on the test surfaces after washing with water was generally similar for cells dried in NaCl or serum. In contrast, when surfaces were washed with household detergent, cells dried in serum were consistently easier to remove from surfaces than those dried in NaCl. Previous workers have also demonstrated that the nature of the suspending fluid in which bacteria are applied to a surface influences the subsequent efficiency with which that surface may be decontaminated. However, these studies have used disinfectants. For example, Bloomfield *et al.* (1994) showed that for *Staph. aureus* cells dried on stainless steel surfaces, the effective concentration of benzalkonium chloride, chlorhexidine and chloroxylenol, was reduced by a factor of 10 for cells dried in 3 % v/v bovine serum albumin, compared to cells dried in tryptone or NaCl. Jones *et al.* (1992) also showed that when dried in bovine serum albumin (0.2 %), the bactericidal effect of hypochlorite against *E. coli* and *Vibrio* was reduced. Log_{10} reductions were < 2 in the presence of albumin, but approximately 6 for cells dried in water. However, it is known that hypochlorite is rapidly inactivated by organic matter.

The influence of the surface type on the efficiency of decontamination was investigated using two contrasting surfaces (ceramic and stainless steel). After water rinsing, recovery of *Staph. aureus* from ceramic surfaces was 2 - 4 fold higher than from stainless steel surfaces, though for *S. enteritidis* there was no consistent trend. Bacterial recoveries after a household detergent rinse were not influenced by the type of surface.

These findings are in agreement with previous observations made by Holah and Thorpe (1990) and Stevens and Holah (1993), who showed that the relative 'cleanability' (with a commercial dish detergent) of a variety of surfaces encountered in the food preparation environment was comparable when the surfaces were new.

The efficiency of removal of *Staph. aureus* and *S. enteritidis* dried on surfaces was also compared. As observed previously (Section 3.2), *Staph. aureus* survival was higher than that of *S. enteritidis* when cells were dried onto surfaces. However, there was no consistent effect of organism type on the proportions of viable organisms which could be removed by rinsing. Although there were differences in the efficiency of decontamination using the two test organisms, which organism was more easily removed depended upon other factors, i.e. the nature of the cell suspending fluid and the presence of detergent etc. Wirtanen and Mattila-Sandholm (1992a), observed that surface decontamination using a variety of agents, including NaOH-HCL, sodium hypochlorite and phosphoric acid was markedly affected by the bacterial species used (*P. fragi*, *Ent. hirae*, *L. monocytogenes* and *B. subtilis*). The numbers of Gram -ve organisms surviving treatment were up to 5 $\log_{10}$ reductions fewer than of *B. subtilis*.

Much of the available information regarding the efficacy of sanitisers recommended for use in the food industry, is based on European suspension tests (Anon, 1988). However, there is some evidence that organisms are less susceptible to the effects of sanitiser treatments when they are surface-bound than when they are in suspension (Mosteler and Bishop, 1993; Bloomfield *et al.*, 1994; Dhir and Dodd, 1995). Holah *et al.* (1990), described a conductance-based surface disinfectant test method in which the

effect of a selection of biocides commonly used in the food industry were evaluated against *Staph. aureus*, *Proteus mirabilis* and *P. aeroginosa* attached to stainless steel. Prior to treatment with the biocides, at the concentrations recommended by the manufacturers, the contaminated surfaces were dipped in bovine serum albumin to simulate the presence of soil on the test surface. The results showed that surface-attached bacteria were at least 10 times more resistant to the biocides tested, which included quaternary ammonium compounds. Wirtanen and Mattila-Sandholm (1992a) carried out similar suspension and surface tests using *P. fragi*, *B. subtilis*, *L. monocytogenes* and *Ent. hirae* suspended in meat soup. They used ATP measurements as indicators of microbial activity but similarly concluded that sanitisers used in the food industry were less effective against surface-attached organisms. However, they appeared to use the sanitisers at low concentrations, and did not indicate whether these were within the range recommended by the manufacturers. Recently, Frank and Chmielewski (1997) demonstrated that the killing efficacy (reduction in bacterial numbers) of quaternary ammonium compounds and chlorine against *Staph. aureus* was reduced by up to 1,000 fold for cells attached to stainless steel, polycarbonate and mineral resin (new and abraded surfaces). However, efficiency was strongly influenced by surface type.

In the limited study of surface decontamination in this thesis, the industrial sanitiser (a quaternary ammonium compound) was highly effective, when used at the concentration recommended by the manufacturer. The number of surface attached *Staph. aureus* and *S. enteritidis* (dried in either serum or NaCl) surviving treatment was below the level of detection (> 4 Log_{10} reductions). Mosteller and Bishop (1993) proposed that a 3 log

cycle reduction in the population of cells attached to a food contact surface was a "reasonable goal" for effective sanitisation.

Since it appears that surface attached organisms are more resistant to disinfectants than organisms in suspension, researchers have proposed testing the activity of disinfectants specifically against surface-bound organisms (Wirtanen and Mattila-Sandholm, 1992b; Bloomfield *et al.*, 1994). An estimate of the activity of surface attached organisms may be obtained by conductance and ATP measurements (see above). However, these cannot be directly related to cell number and in other studies, organisms were recovered from test surfaces prior to enumeration. However, Holah *et al.* (1988) reported that the use of swabbing for removing bacteria remaining on surfaces after cleaning, gave highly variable data. The results obtained in this section of the thesis suggest that the *in situ* NBT method could form the basis of a new method for assessing the efficacy of detergents against surface-attached organisms. The method gives a precise estimate of the number of surviving bacteria. However, it is recognised that the disinfection test might require modifications, e.g. by incorporation of a disinfectant inactivator in the final rinse solution prior to overlaying with agar. This possible requirement was not assessed, as the main aim was to further demonstrate the application of *in situ* NBT detection. It was, though, shown that nutrient agar could be replaced by gelatin agar in the *in situ* method, so as to avoid any heat stress due to the higher temperature (45 °C) required using molten agar. The *in situ* NBT method might also be readily adapted to tests of the decontamination of soiled surfaces. Soil is known to interfere with the efficacy of certain chemical treatments (Ak *et al.* 1994b). Since the initial publication of the *in situ* method (Barnes *et al.* 1996), Welker *et al.* (1997) used a similar approach to

determine the 'cleanability' of plastic and wooden cutting boards. Bacteria remaining on surfaces after washing were detected by overlaying the surface with agar containing X-gal (a chromogenic β - galactosidase substrate). After incubation at 37 °C for 48h, blue-coloured colonies were counted directly on the test surfaces. However, this method is only suitable for detecting bacteria possessing β - galactosidase and validation of the technique would require a study of the induction of β - galactosidase by stressed, surface-attached bacteria.

SECTION IV.

The development of novel decontamination methods for food and food-contact surfaces using combined trisodium phosphate, osmotic shock and lysozyme treatments.

4.1. INTRODUCTION

A variety of chemical agents have been considered for their potential use in the decontamination of meats and poultry (see Section 1.8). These include, parabens (Jay, 1996), sorbic acid (Robach and Sofos, 1982) and organic acids such as acetic and lactic acid (Dickson, 1992; Dickson and Siragusa, 1994; van Netten *et al.*, 1995; Monk *et al.*, 1995), which are already widely used as preservatives in food products (Cherrington *et al.*, 1991), and chlorine and hypochlorites, which are useful in reducing the levels of spoilage and pathogenic microorganisms within the food processing environment (Zottola and Sasahara, 1994).

Despite the apparent success of some of these agents in reducing contamination, there are frequently disadvantages associated with their use. These include inhibition of antibacterial activity due to the presence of fatty and other organic deposits; deterioration of product quality due to bleaching (chlorine and hypochlorite); tainting of food products; the presence of toxic residues of antibacterial agents; and consumer resistance to the use of chemicals as food decontaminating agents (Gould, 1996). Consequently, there is considerable interest in the development of naturally derived food decontaminating agents. The bacteriocin nisin and the enzyme lysozyme are considered to have the greatest potential in this area. Their advantages include a high stability and activity under a wide range of physical conditions (Salton, 1957; Hurst, 1981).

Lysozyme is already used in the control of certain Gram +ve foodborne pathogens and spoilage organisms. It hydrolyses the β1-4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine residues of cell wall peptidoglycan. This results in the loss of rigidity of the cell wall and subsequent cell lysis. Gram -ve organisms are much less susceptible to lysozyme activity, because in Gram -ve bacteria, peptidoglycan occurs as a discrete layer protected from the environment by an outer cell membrane; the lysozyme molecule is too large to penetrate the undamaged outer membrane and gain access to the peptidoglycan (see Section 1.8.2.5.1).

It has been previously reported that the spectrum of lysozyme activity may be extended to encompass Gram -ve organisms, by disruption of the outer cell membrane (Noller and Hartsell, 1960). However many of the membrane disruption methods proposed are not appropriate to the food industry (see Section 1.8.2). The work described in this section of the thesis investigates the use of NaCl-induced osmotic shock and TSP treatments to disrupt the outer membrane and increase lysozyme sensitivity.

NaCl has historically been utilised within the food industry as a preservative (Gould, 1996), particularly for meats. The addition of NaCl to foods increases water activity and, depending upon the concentration added, may prevent growth (Houtsma *et al.*, 1996). Upon exposure to high concentrations of NaCl, cells may undergo plasmolysis, resulting in a decrease in cytoplasmic water activity and impaired cellular activity (Csonka, 1989). In previous work carried out at King's College (Chatzopoulou *et al.*, 1993), NaCl-induced osmotic shock treatments were combined with cold shock and/or exposure to lysozyme to kill Gram -ve bacteria. The most effective procedure involved exposure of cells to NaCl (0.4 - 1.2 M) followed by rapid dilution in deionised water containing lysozyme; presumably, during dilution, the rapid influx of water into cells led to disruption of the outer membrane sufficient to allow entry of lysozyme.

Phosphates have also been used for many years in the food industry to protect or enhance the flavour of meat products (Lee *et al.*, 1994a). However, their antimicrobial properties are now becoming recognised and they are increasingly used as antimicrobial agents. Trisodium phosphate (TSP) has recently been approved for use during poultry processing by the USDA (1982; 1994) (Giese, 1993c). Its use, particularly in relation to killing of *Salmonella,* is the subject of a recent patent (Bender & Brotsky, 1992). The principal claim of the patent is 'a process for treating poultry carcasses .....with a treatment solution having a pH above about 11.5 and containing trialkali metal orthophosphate in an amount from about 4 % to about 12 % based on the weight of the solution'. Rhône Poulenc market the TSP poultry treatment under the name of AvGard.

The efficacy of TSP as a bactericidal agent for the decontamination of chicken carcasses has been the subject of several publications (Kim & Slavik, 1994a; Kim *et al.*, 1994; Lillard, 1994; Coppen, 1993; Slavik *et al.*, 1994; Hwang & Beuchat, 1995). TSP has also been evaluated for the treatment of beef (Kim & Slavik, 1994a; Dickson *et al.*, 1994) and salad vegetables (Zhuang & Beuchat, 1996). At a concentration of 10 % (w/w), TSP reduced the numbers of *E. coli* O157:H7 and *S. typhimurium* by 1.4 and 0.9 Log_{10} cycles respectively on fat surfaces of beef tissue, though significantly lower reductions (0.9 and 0.5 Log_{10} cycles respectively) were observed on fascia (Kim and Slavik, 1994b). A subsequent study on beef (Dickson *et al.*, 1994) showed similar results and again bacterial reductions were greater on adipose tissue. Treatment of chicken carcasses with TSP (10 % w/w) at 10 or 50 °C, reduced *Salmonella* contamination by 1.6 to 1.8 Log_{10} cycles per carcass (Slavik *et al.* 1994; Kim *et al.* 1994); and in a scanning electron microscopic study, Kim and Slavik (1994a) showed that TSP (10 % w/v) treatment of chicken skin effectively removed inoculated *Salmonella* from the surface. Interestingly, although *Campylobacter* was also killed by the 50 °C treatment (1.2 to 1.5 Log_{10} cycles reduction), it was resistant at 10 °C (Slavik *et al.*, 1994). Recently, 10 % TSP has been found to be highly effective in killing *Salmonella montevideo* on the surface of tomatoes and reducing cell numbers in artificially contaminated tomato core tissue (Zhuang & Beuchat, 1996). In a further potential application of TSP, to the disinfection of food-preparation surfaces, Somers *et al.* (1994) reported that *S. typhimurium* and cells of other Gram -ve species attached to stainless steel or buna-N rubber surfaces were killed by exposure to 1 % (w/v) TSP; however, attached cells appeared more resistant than suspended cells.

Possible modes of action of TSP against microorganisms include: exposure to high pH, particularly of cell membrane components (Mendonca *et al.*, 1994); sequestration of metal ions (Lee *et al.*, 1994a); and a role as a surfactant, enhancing detachment of bacteria from food surfaces (Kim & Slavik, 1994a, 1994b). However, Lillard (1994) has suggested that the apparent reduction in counts of *Salmonella,* artificially inoculated on broiler

carcasses, may be an artefact of the high pH of recovery media due to TSP contamination. They showed that salmonellae were recoverable from carcasses inoculated with only 10^2 cfu, if the carcasses were adequately rinsed with water after treatment and the recovery medium was buffered. Nevertheless, there is considerable evidence to suggest that high concentrations of TSP are effective in killing Gram -ve bacteria including organisms attached to chicken carcasses.

The principal disadvantages of the TSP decontamination procedure are the requirement for a high TSP concentration ($\geq$ 0.4 M), and thus a high pH (12.0), which may reduce its acceptability for the treatment of poultry and other foods. In addition, the TSP procedure is reported to be ineffective against Gram +ve spoilage bacteria and perhaps even certain Gram -ve bacteria, for example *Campylobacter* species (Slavik *et al.* 1994). Dickson *et al.* (1994) showed that Gram +ve bacteria are generally more resistant than Gram -ve bacteria to the effects of alkaline antibacterials.

However, it is possible that although high concentrations of TSP may be needed for cell-killing, much lower concentrations may disrupt the outer membrane of Gram -ve bacteria, since a major aspect of TSP toxicity appears related to its surfactant property. Thus, TSP might act synergistically with osmotic shock/lysozyme treatments. This possibility was investigated in the work described in this section of the thesis. Effects on suspended bacteria are considered in Section 4.2 and on bacteria attached to foods (principally chicken skin) in Section 4.3. Section 4.3 also reports some work on the decontamination of food contact surfaces.

4.2. The effect of combined trisodium phosphate, osmotic shock and lysozyme treatments on suspended microorganisms.

4.2.1. INTRODUCTION

The hypothesis that TSP might promote susceptibility of Gram -ve bacteria to lysozyme and/or osmotic shock was initially tested using cells in suspension. Previously, Chatzopoulou (1991) showed that kills of *E. coli, P. fluorescens, S. enteritidis* and *S. typhimurium,* by osmotic shock plus lysozyme treatment were > 99.9 %, under optimal conditions; however, conditions giving optimal kills varied with the test organism used. Osmotic shock was achieved by first suspending cells in NaCl (hyperosmotic shock) followed by dilution in deionised water containing lysozyme (hypoosmotic shock). Two contrasting conditions giving high cell kills were identified. The first procedure (Procedure 1) involved incubating cells for a short period (1-10 min) in hypertonic (low water activity, a_w) medium at 10-37 °C, followed by the transfer of cells to deionised water containing lysozyme. High cell kills were crucially dependent upon the inclusion of organic nutrients in the hypertonic medium and the procedure was more effective at 37 °C than at lower temperatures. The second procedure (Procedure 2) involved transferring cells from ambient temperature to a hypertonic (low a_w) medium at low temperature (0 to 10 °C) for 30 min, followed by dilution in deionised water or deionised water containing lysozyme. Whilst under optimal conditions, high kills of all the organisms tested could be achieved, neither procedure was considered ideal for food processing. Procedure 1 relied on the inclusion of organic nutrients (brain heart infusion or glucose) in the hypertonic treatment step and Procedure 2 was ineffective against certain bacteria, particularly *Campylobacter.* In addition, the effectiveness of both procedures was greatly reduced for cells attached to chicken skin.

The methodology adopted in this section was based on previous work by Chatzopoulou (1991), which indicated that: (i) early stationary phase cells were the most resistant to osmotic shock/lysozyme treatments; (ii) a concentration of 0.8 M NaCl gave maximal or near-maximal killing for all the Gram -ve organisms tested; (iii) exposure to hyper- and hypo-osmotic shocks for 10 and 30 min respectively was adequate to allow access of lysozyme to Gram -ve cells and longer treatment times would be commercially

unacceptable; and (iv) 20 μg ml^{-1} lysozyme gave close to optimal cell kills for suspended cells of osmotically-shocked Gram -ve bacteria.

In the present study, the test Gram -ve organisms used were: *C. jejuni, P. fluorescens, S. enteritidis* and *E. coli.* These organisms were selected because they are commonly encountered food-borne pathogens or meat spoilage organisms (*P. fluorescens*). In addition, two Gram +ve organisms were tested; these were *L. monocytogenes* and *Staph. aureus*. *Listeria monocytogenes* and *Staph. aureus* were also chosen for use in the present study because they are commonly encountered food-borne pathogens.

This section (Section 4.2.) of the thesis describes experiments conducted to determine the effect of combined TSP and osmotic shock/lysozyme treatments on suspended cells. Once conditions had been optimised for suspended cells, it was anticipated that the combined treatments could be used on cells attached to surfaces. However, it was recognised that cells in suspension are much more susceptible to bactericidal treatments than cells attached to surfaces (Wilson, 1996).

4.2.2. RESULTS

Except where stated, all experiments were carried out in duplicate or triplicate and the data shown are for representative experiments. The initial and final pH values of treatment solutions were measured in nearly all cases; typical data are given for each type of experiment.

4.2.2.1. The effect of TSP exposure on survival of Gram -ve and Gram +ve bacteria.

The effect of exposing bacterial cell suspensions to TSP for 10 min is shown in Table 4.2.1. The concentration required to reduce populations of Gram -ve organisms (*C. jejuni, E. coli, P. fluorescens* and *S. enteritidis*) by more than 90% was between 0.002 and 0.005 M. In contrast, > 0.05 M TSP was required to cause similar kills of the Gram

+ve organisms (*L. monocytogenes* and *Staph. aureus*). TSP was more effective against Gram +ve organisms at 37 than at 4 °C. However, kills of the Gram-ve organisms were similar at the two temperatures tested.

Table 4.2.1. The effect of TSP concentration on the survival of Gram -ve and Gram +ve bacteria.

Cells were diluted by a factor 1:100 into TSP solutions at 37 °C or 4 °C and incubated for 10 min. The initial cell concentrations in TSP solutions were approximately 2.5 x 10^7 cfu ml^{-1}. There was no killing effect when cells were diluted into deionised water.

Organism	% Survival at 37 °C				
	Trisodium phosphate concentration (M)				
	0.002	0.005	0.01	0.05	0.10
E. coli	100	ns	ns	ns	ns
P. fluorescens	99	ns	ns	ns	ns
S. enteritidis	81	0.05	ns	ns	ns
C. jejuni	80	ns	ns	ns	ns
L. monocytogenes	100	100	100	21	ns
Staph. aureus	100	100	86	11	ns

Organism	% Survival at 4 °C				
	Trisodium phosphate concentration (M)				
	0.002	0.005	0.01	0.05	0.10
E. coli	50	ns	ns	ns	ns
P. fluorescens	90	ns	ns	ns	ns
S. enteritidis	56	ns	ns	ns	ns
C. jejuni	65	4.1	ns	ns	ns
L. monocytogenes	100	100	100	89	8.5
Staph. aureus	100	100	92	75	73

ns, no survivors detected; cell kill > 99.7 %.

4.2.2.2. The relationship between TSP concentration, pH and killing of *E. coli.*

Cell killing due to TSP may be influenced by both its surfactant nature and the alkaline pH of TSP solutions. The relationship between TSP concentration, cell killing and the pH values of *E. coli* cell suspensions are shown in Table 4.2.2; the pH values are those recorded for cell suspension plus TSP and are slightly lower than for pure TSP solutions. Cell kills became significant at approximately 0.0025 M where the pH value was above 10. Similarly, when a 0.05 M TSP solution was gradually acidified, and used in experiments at various pH values, higher numbers of cells survived at pH values below approximately 10 (Table 4.2.3).

Table 4.2.2. The relationship between TSP concentration, pH and killing of *E. coli*.

Cells were diluted by a factor of 1:100 into TSP solutions at 37°C and incubated for 10 min. The initial viable count was 2.3×10^7 cfu ml^{-1}.

TSP concentration (M)	pH Value	% Cell Survival
0.0010	8.35	100
0.0020	8.85	100
0.0025	10.48	45
0.0030	10.96	7.3
0.0040	11.30	4.6
0.0050	11.41	ns

ns, no survivors detected; cell kill > 99.7 %.

Table 4.2.3. The relationship between pH and killing of *E. coli* in acidified 0.05M TSP at 37 °C.

The pH of a 0.05M TSP solution (initial pH 12.5) was modified by the addition of concentrated HCl. Cells were exposed to the TSP solution for 10 minutes. The initial cell concentration was 2.7×10^7 cfu ml^{-1}.

pH	% cell survival
7.0	100
8.0	99
9.0	97
10.0	82
11.0	ns
12.5	ns

ns, no survivors detected; cell kill > 99.7 %.

4.2.2.3. The effect of osmotic shock and lysozyme treatment on the survival of Gram -ve bacteria.

A 10 min exposure to lysozyme (20 $\mu g\ ml^{-1}$) did not reduce the viable count of *C. jejuni, P. fluorescens, S. enteritidis* and *E. coli* suspended in deionised water at 4 or 37 °C. Similarly, osmotic shock treatment, i.e. exposure to 0.8 M NaCl followed by dilution (1:100) in deionised water, had no effect on the viability of *C. jejuni* and *P. fluorescens.* However, *S. enteritidis* and *E. coli* appeared susceptible. Reductions in counts were approximately 60 % for *E. coli* at both temperatures and 60 and 30 % for *S. enteritidis* at 4 and 37 °C respectively. Consistent with the results of Chatzopoulou (1991), combining osmotic shock and lysozyme treatments, by suspending cells in NaCl solutions and diluting in deionised water containing lysozyme, markedly enhanced the killing of *E. coli* (Table 4.2.4). At 37 °C, the extent of killing was dependent upon the magnitude of the osmotic shock in the experiments, and increased from 89 % to > 99 % as the NaCl concentration used was increased from 0.4 to 1.2 M. Chatzopoulou (1991) reported that for a variety of Gram -ve organisms, 0.8 M NaCl was the optimal concentration for osmotic shock treatment, and this concentration was used in further experiments with *E. coli* at 4 °C and with other organisms at both 4 and 37 °C (Tables 4.2.3 and 4.2.4).

At 37 °C, *C. jejuni, P. fluorescens* and *S. enteritidis* were more resistant than *E. coli* to the combined osmotic shock lysozyme treatment; nevertheless, the approximate reduction in counts for *P. fluorescens* and *S. enteritidis* were 90 % and 60 % respectively; *C. jejuni* cells were much more resistant, with a reduction of only 5 %. At 4 °C, the combined lysozyme osmotic shock treatment was effective in reducing the numbers of viable *E. coli* cells to below 1 %. At this lower temperature, *C. jejuni, P. fluorescens* and *S. enteritidis* were again more resistant than *E. coli.* However, kills were higher at 4 °C than at 37 °C; for *P. fluorescens,* cell kill increased to 97 % and for *S. enteritidis* to 83 %.

Campylobacter jejuni cells were again the most resistant, with a reduction in viable count of only 10 %.

4.2.2.4. The combined effect of TSP, osmotic shock and lysozyme treatment on the survival of Gram -ve bacteria.

The effect of TSP on cell killing by lysozyme and/or osmotic shock treatments was determined for a range of Gram -ve bacteria at 4 and 37 °C. The TSP concentrations used were in the range 0.001M to 0.010 M . The results are given in Tables 4.2.4 and 4.2.5. The majority of the data selected for inclusion in these tables show the effect of TSP on cell killing at concentrations which in the absence of lysozyme and osmotic shock had no or little effect on cell viability.

At 37 °C, treatment of *S. enteritidis, E. coli, C. jejuni* and *P. fluorescens* with low concentrations of TSP (0.001 and 0.002 M), markedly enhanced their subsequent susceptibility to lysozyme. *Escherichia coli* was particularly sensitive and cell kill was increased from 0 % (TSP and lysozyme treatment only) to 97 % for the combined treatment. Low concentrations of TSP also markedly enhanced cell killing by osmotic shock (0.8 M NaCl). Using 0.001 M TSP, the numbers of *P. fluorescens* cells surviving treatment fell from 100 % to < 1 %. Similarly, using 0.002 M TSP, survival of *E. coli* and *C. jejuni* fell from 33 % and 100 % (osmotic shock alone) to 4 and < 1 %, respectively. *Salmonella enteritidis* was notably less susceptible. However the most successful killing effect for all test organisms was achieved when treatment with TSP was combined with both osmotic shock and exposure to lysozyme. This combined treatment with TSP concentrations ≤ 0.002 M, reduced cell survival to below 99.7% for all organisms tested.

Table 4.2.4. The effect of TSP, osmotic shock and lysozyme on the survival of Gram -ve bacteria at 37°C.

Cells were diluted by a factor of 1:100 into TSP, NaCl, TSP + NaCl solutions and deionised water at 37°C and incubated for 10 min. Cells were then diluted by a factor of 1:100 into deionised water or lysozyme solutions at 37°C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5 x 10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH *	% cell survival	
				- lysozyme	+ lysozyme
E. coli	none	none	7.01	100	87
	0.4	none	6.57	73	11
	0.8	none	6.39	33	0.59
	1.2	none	6.26	13	ns
	none	0.002	10.46	100	2.3
	0.8	0.002	9.56	3.8	ns
P. fluorescens	none	none	7.12	100	100
	0.8	none	6.36	100	12
	none	0.001	7.59	100	100
	none	0.002	9.07	99	66
	0.8	0.001	6.81	ns	ns
S. enteritidis	none	none	7.01	100	99
	0.8	none	6.28	86	41
	none	0.001	8.70	91	71
	none	0.002	10.69	63	28
	none	0.005	10.87	1.0	ns
	0.8	0.001	8.42	74	ns

Continued

Table 4.2.4. Continued.

C. jejuni	none	none	7.34	100	100
	0.8	none	6.62	100	95
	none	0.001	8.89	100	76
	none	0.002	9.89	100	32
	none	0.005	10.67	ns	ns
	0.8	0.001	7.38	95	33
	0.8	0.002	9.42	ns	ns

ns, no survivors detected; cell kill > 99.7%

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water.

At 4 °C, treatment with TSP at concentrations (0.001 or 0.002 M) which had relatively little effect on viability when used separately, were highly effective against all the test organisms (cell kills ≥ 97 %), when combined with osmotic shock. TSP treatment also enhanced killing by lysozyme, but to a lesser extent. Using ≤ 0.002 M TSP, the TSP plus osmotic shock and lysozyme treatment reduced the numbers of *S. enteritidis, E. coli* and *P. fluorescens* cells to an undetectable level (cell kill > 99.7 %). However, survival of *C. jejuni* was approximately 2 %. Raising the TSP concentration to 0.005 M did reduce survival of *C. jejuni* to an undetectable level. However, at this concentration, TSP treatment alone caused a significant kill (96 %). Comparison of the data at 4 °C and 37 °C (Tables 4.2.5 and 4.2.4, respectively), shows that at the lower temperature, *C. jejuni* was significantly more resistant to the effect of TSP and combined TSP/osmotic shock procedure, with or without lysozyme.

Table 4.2.5. The effect of TSP, osmotic shock and lysozyme on the survival of Gram -ve bacteria at 4 °C.

Cells were diluted by a factor of 1:100 into TSP, NaCl, TSP + NaCl solutions and deionised water at 4 °C and incubated for 10 min. Cells were then diluted by a factor of 1:100 into deionised water or lysozyme solutions at 4 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5 x 10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
E. coli	none	none	6.80	100	100
	0.8	none	6.36	31	ns
	none	0.002	9.33	51	10
	none	0.005	11.30	ns	ns
	0.8	0.002	9.54	ns	ns
P. fluorescens	none	none	7.04	100	100
	0.8	none	6.36	100	41
	none	0.001	7.54	65	85
	none	0.002	9.26	99	56
	0.8	0.001	7.01	ns	ns
S. enteritidis	none	none	7.15	100	100
	0.8	none	6.32	29	17
	none	0.001	7.40	80	77
	none	0.002	8.42	52	34
	none	0.005	11.26	1.0	ns
	0.8	0.001	6.77	9	ns
	0.8	0.002	8.80	10	ns
	0.8	0.005	10.29	1.0	ns

Continued

Table 4.2.5. Continued.

C.jejuni	none	none	7.42	100	100
	0.8	none	6.64	100	90
	none	0.001	7.84	100	67
	none	0.002	8.68	100	89
	none	0.005	10.05	3.5	3.1
	0.8	0.001	7.66	95	33
	0.8	0.002	9.00	3.0	2.6
	0.8	0.005	9.68	ns	ns

ns, no survivors detected; cell kill > 99.7 %.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water.

4.2.2.5. The effect of osmotic shock and lysozyme treatment on the survival of Gram +ve bacteria.

The effects of lysozyme (20 μg ml^{-1} and 100 μg ml^{-1}) on the survival of *L. monocytogenes* and *Staph. aureus* (Table 4.2.6) was determined at 37 °C. To achieve a total kill of *Staph. aureus* it was necessary to use the higher concentration of lysozyme (100 μg ml^{-1}) for 2 h. However, *L. monocytogenes* was relatively sensitive, even to the lower concentration of lysozyme (20 μg ml^{-1}), and no survivors were detected after 1 h incubation. The exposure time and lysozyme concentration required to kill *Staph. aureus* cells showed that, in the context of the rapid killing procedures being developed in this section of the thesis, killing by lysozyme alone would not be significant. This was confirmed in subsequent experiments (see Tables 4.2.7 and 4.2.8). However, a 30 min exposure to lysozyme (20 μg ml^{-1}) reduced the viable count of *L. monocytogenes* by 99 % and 80 % at 37 and 4 °C, respectively. It would be predicted that the activity of hen egg lysozyme would be greater at the higher temperature.

Osmotic shock treatment, i.e. exposure to 0.8 M NaCl followed by dilution in (1:100) in deionised water, had no effect on the viability of *L. monocytogenes* at 37 and 4 °C or of *Staph. aureus* at 4 °C (Tables 4.2.7 and 4.2.8). However, at 37 °C, the survival of *Staph. aureus* was reduced by approximately 40 %. Combining osmotic shock and lysozyme treatments did not enhance cell killing, i.e. osmotic shock did not increase the susceptibility of *L. monocytogenes* to lysozyme and the small cell kill of *Staph. aureus* observed following osmotic shock treatment at 37 °C was not increased by the presence of lysozyme.

Table 4.2.6. The effect of time of exposure to lysozyme on the survival of cell suspensions of *L. monocytogenes* and *Staph. aureus*.

Cells were diluted by a factor of 1:100 into lysozyme solutions at 37 °C and incubated for up to 24 h. The initial cell concentrations in lysozyme solutions were approximately 2.5 x 10^7 cfu ml^{-1}.

Organism	Lysozyme	% cell survival			
	(μg ml -1)	5 min	1 h	2 h	24 h
L. monocytogenes	20	38	ns	ns	ns
	100	28	ns	ns	ns
Staph. aureus	20	100	100	100	ns
	100	85	52	ns	ns

ns, no survivors detected; cell kill > 99.7 %.

4.2.2.6. The combined effect of TSP, osmotic shock and lysozyme treatment on the survival of Gram +ve bacteria.

The effect of TSP on cell killing of *L. monocytogenes* and *Staph. aureus* by lysozyme and/or osmotic shock treatments was determined at 4 and 37 °C (Tables 4.2.7 and 4.2.8). At both temperatures, 0.005 M TSP had little or no effect on cell viability. However, cell kills increased progressively as the TSP concentration was increased. To obtain kills of > 25 %, TSP concentrations of 0.05 M (37 °C) or 0.10 M (4 °C) were required.

At 37 °C, combining TSP treatment with osmotic shock (0.8 M NaCl) increased cell kills significantly. For example, using 0.01 M TSP, cell kill was increased from 12 to 73 % and from 23 to 99.7 % for *L. monocytogenes* and *Staph. aureus*, respectively. As expected, the inclusion of lysozyme (20 μg ml^{-1}) in TSP or TSP/osmotic shock killing procedures, had relatively little effect on the survival of *Staph. aureus*, but markedly enhanced kills with *L. monocytogenes*. At only 0.005 M TSP, with this organism, no survivors were detected following the combined TSP/osmotic shock/ lysozyme treatment.

Table 4.2.7. The effect of TSP, osmotic shock and lysozyme on the survival of Gram +ve bacteria at 37 °C.

Cells were diluted by a factor of 1:100 into TSP, NaCl (0.8 M), TSP + NaCl or deionised water at 37 °C and incubated for 10 min. They were then diluted by a factor of 1:100 into deionised water or lysozyme solution at 37 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5 x 10^7 cfu ml^{-1}

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
L. mono-cytogenes	none	none	6.89	100	1.2
	0.8	none	6.32	100	4.9
	none	0.005	10.81	88	3.4
	none	0.010	11.54	88	8.1
	none	0.050	12.22	4.0	ns
	none	0.100	12.50	ns	ns
	0.8	0.005	10.35	27	ns
	0.8	0.010	10.80	10	ns
	0.8	0.050	11.87	ns	ns
Staph. aureus	none	none	6.99	100	100
	0.8	none	6.32	64	77
	none	0.005	9.92	100	53
	none	0.010	11.51	77	78
	none	0.050	12.23	ns	ns
	0.8	0.005	9.66	33	17
	0.8	0.010	9.88	ns	ns
	0.8	0.050	11.65	ns	ns

ns, no survivors detected; cell kill > 99.7%.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or deionised water.

At 4 °C *L. monocytogenes* and *Staph. aureus* showed greater resistance to TSP (Table 4.2.8) than at 37 °C (Table 4.2.7). Nevertheless, in combined TSP/osmotic shock killing procedures, there was still a marked synergy between the effects of TSP and osmotic shock, particularly for *L. monocytogenes*. However, at equivalent TSP concentrations, kills were in all cases lower than at 37 °C and with 0.05 M (*L. monocytogenes*) or 0.10 M (*Staph. aureus*) TSP, maximum kills were 85 - 87 %. As at 37 °C, lysozyme did not greatly increase kills with *Staph. aureus*, but enabled high kills (> 99.7 %) of *L. monocytogenes* to be achieved at 0.05 M TSP without osmotic shock, or at only 0.005 M TSP with osmotic shock.

Table 4.2.8. The effect of TSP, osmotic shock and lysozyme on the survival of Gram +ve bacteria at 4 °C.

Cells were diluted by a factor of 1:100 into TSP, NaCl (0.8 M), TSP + NaCl solutions or deionised water at 4 °C and incubated for 10 min. They were then diluted by a factor of 1:100 into deionised water or lysozyme solution at 4 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5 x 10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
L. monocytogenes	none	none	7.06	100	20
	0.8	none	6.26	100	20
	none	0.005	11.38	100	33
	none	0.010	11.82	100	3.2
	none	0.050	12.34	100	ns
	none	0.100	12.46	9.1	ns
	0.8	0.005	10.62	77	ns
	0.8	0.010	11.24	53	ns
	0.8	0.050	11.93	15	ns
Staph. aureus	none	none	7.28	100	100
	0.8	none	6.33	100	100
	none	0.010	11.51	100	100
	none	0.050	12.28	85	65
	none	0.100	12.44	83	40
	0.8	0.010	11.29	100	91
	0.8	0.050	11.85	94	55
	0.8	0.100	12.23	13	20

ns, no survivors detected; cell kill > 99.7 %.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water.

4.2.2.7. The effect of organic load on TSP and combined TSP/osmotic shock/lysozyme treatments for killing Gram -ve and Gram +ve cells.

The influence of an organic load (50 % v/v inactivated newborn calf serum) on killing by TSP alone and in combination with osmotic shock and/or lysozyme treatments at 37 and 4 °C, was determined for a range of Gram -ve (*C. jejuni, E. coli, P. fluorescens* and *S. enteritidis*; Tables 4.2.9 and 4.2.10) and Gram +ve organisms (*L. monocytogenes* and *Staph. aureus*; Tables 4.2.11 and 4.2.12).

4.2.2.7.1. Effect of organic load on killing of Gram -ve cells by TSP.

At both 4 and 37 °C, the presence of organic load reduced killing of Gram -ve cells by TSP (c.f. Table 4.2.1 and Tables 4.2.9 and 4.2.10). In the absence of serum, 0.005 M TSP killed 96 to 100 % of suspended cells of the four Gram -ve species tested (Table 4.2.1); however, in the presence of 50 % serum, kills were ≤ 44 %.

Significantly, in the presence of serum, the pH of TSP solutions decreased (c.f. Table 4.2.2 and Tables 4.2.9 - 4.2.12); for example, the pH of cell suspensions containing 0.005 M TSP was >11 without serum, but only approximately 8.5 in the presence of serum.

4.2.2.7.2. Effect of organic load on killing by osmotic shock and/or lysozyme treatments.

Lysozyme treatment of the Gram -ve test organisms suspended in 50 % serum, resulted in variable cell kills. At 37 °C, lysozyme in the presence of serum, reduced the viability of *E. coli, P. fluorescens* and *S. enteritidis* cell suspensions by ≤ 5 %; however, kills of *C. jejuni* were 25 %. At 4 °C, *C. jejuni*, was again the most susceptible organism with a cell kill of 34 %. In comparison with results obtained in the absence of organic load (Tables 4.2.4 and 4.2.5), it appears that the presence of serum has little effect on the susceptibility of Gram -ve cells to lysozyme.

Killing by osmotic shock (0.8 M NaCl) was also little affected by the presence of serum. Kills of the test organisms were relatively low (up to 70 %) and generally similar to those in the absence of serum.

In combined osmotic shock/lysozyme treatments, in the absence of serum, kills varied widely for the test organisms, from < 10 % for *C. jejuni* to > 99.5 % for *E. coli*, at both 4 and 37 °C. In general, serum reduced cell kills which were between 47 and 96 % at 37 °C, and 10 and 80 % at 4 °C. However, for *C. jejuni*, killing was greater in the presence of serum. It is possible that serum may increase the osmotic shock for *C. jejuni* to a value closer to the optimum. However, it is also possible that the serum used contained anti-*Campylobacter* factors, including antibodies which might increase cell death or cause cell aggregation.

4.2.2.7.3. Effect of organic load on killing of Gram -ve cells by osmotic shock/lysozyme treatment combined with TSP.

Cell killing by TSP alone was greatly reduced, at any particular TSP concentration, by the presence of serum (Section 4.2.2.7.1), which reduced the pH of TSP solutions. Therefore it was expected that in combined killing procedures involving TSP, cell kills would be similarly reduced by the inclusion of serum. In the absence of serum, the extent of cell killing by TSP (0.001 - 0.005 M) plus lysozyme was species dependent with high kills (> 95 %) being observed for *S. enteritidis*, *E. coli*, and *C. jejuni* and low kills (< 50 %) for *P. fluorescens* (Tables 4.2.4 and 4.2.5). At equivalent TSP concentrations, in the presence of serum, high cell kills at 37 °C were found only for *E. coli*. Increasing the TSP concentration 0.01 M had relatively little effect. At 4 °C (Table 4.2.10), the protective effect of serum was even more marked. Kills were < 20 %, except with *C. jejuni* (60 % kill at 0.01 M TSP); however, with this organism there was no evidence for any synergic interaction between TSP and lysozyme treatments.

The killing efficiency of the TSP plus osmotic shock procedure was less affected by the presence of serum than the TSP plus lysozyme procedure. At both 4 and 37 °C, high maximum kills (≥ 99 %) were obtained for all test organisms, except *S. enteritidis* at 4 °C (41 % kill). However, to obtain maximum kills, TSP concentrations had to be increased to up to 0.01 M.

In the absence of serum, the highest cell kills were obtained by combining TSP with both osmotic shock and lysozyme treatments. At both 4 and 37 °C, the data obtained in the presence of serum also clearly show a synergistic interaction between the three components of this combined treatment (except for *C. jejuni* at 4 °C, where kills were similar in the presence and absence of lysozyme). In the presence of serum, the maximum kills obtained by TSP plus osmotic shock and lysozyme treatments were generally ≥ 93 % (0.005 M TSP) or > 99.7 % (0.10 M TSP). The exceptions were *C.*

jejuni and *S. enteritidis* at 4 °C , for which kills were low using 0.005 M TSP. However, kills of > 99.7 % and 90 %, respectively, were obtained using 0.01 M TSP. Thus, although it appears that high concentrations of serum do protect Gram -ve cells during cell killing procedures involving TSP, this effect can be largely overcome by increasing the TSP concentration to 0.005 or 0.01 M. Importantly, this does not lead to an increase in the pH of test TSP suspensions due to the buffering effect of the serum. High kills were obtained in all cases at a pH of approximately 9.

Table 4.2.9. The effect of TSP, osmotic shock and lysozyme on the survival of Gram -ve bacteria at 37 °C in the presence of 50 % v/v serum.

Cells were diluted by a factor of 1:100 into TSP + serum, NaCl + serum, TSP + NaCl + serum or deionised water + serum at 37 °C. All suspensions were incubated for 10 min. Cells were then diluted by a factor of 1:100 into deionised water or lysozyme solutions at 37 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5 x 10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
E. coli	none	none	7.54	100	100
	0.8	none	7.00	100	15
	none	0.002	8.01	40	2.8
	none	0.005	8.35	56	5.3
	none	0.01	9.27	84	ns
	0.8	0.002	7.88	100	ns
	0.8	0.005	8.56	10	ns
	0.8	0.01	9.20	ns	ns
P. fluorescens	none	none	7.20	100	97
	0.8	none	6.94	46	6.8
	none	0.001	7.45	99	88
	none	0.002	7.72	81	72
	none	0.005	8.59	74	70
	0.8	0.001	7.15	48	2.7
	0.8	0.002	7.43	34	3.4
	0.8	0.005	8.45	ns	ns

Continued

Table 4.2.9. Continued

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
S. enteritidis	none	none	7.22	100	100
	0.8	none	6.00	32	3.8
	none	0.005	8.35	100	71
	none	0.01	9.32	90	82
	0.8	0.002	7.35	35	1.1
	0.8	0.005	8.10	1.8	1.1
	0.8	0.01	8.91	ns	ns
C. jejuni	none	none	7.13	100	75
	0.8	none	6.68	75	53
	none	0.005	8.59	95	88
	none	0.01	9.56	21	23
	0.8	0.005	7.88	37	37
	0.8	0.01	9.14	ns	ns

ns, no survivors detected; cell kill > 99.7 %.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water (all containing 50 % v/v serum)

Table 4.2.10. The effect of TSP, osmotic shock and lysozyme on the survival of Gram -ve bacteria at 4 °C in the presence of 50 % v/v serum.

Cells were diluted by a factor of 1:100 into TSP + serum, NaCl + serum, TSP + NaCl + serum or deionised water + serum at 37 °C. All suspensions were incubated for 10 min. Cells were then diluted by a factor of 1:100 into deionised water or lysozyme solutions at 37 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5×10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
E. coli	none	none	7.42	100	100
	0.8	none	7.18	100	20
	none	0.002	7.87	100	100
	none	0.005	8.92	100	100
	none	0.01	9.66	100	94
	0.8	0.002	7.18	53	10
	0.8	0.005	8.37	25	5.2
	0.8	0.01	9.34	1.0	ns
P. fluorescens	none	none	7.18	100	100
	0.8	none	6.93	66	14
	none	0.001	7.43	100	100
	none	0.002	7.74	100	91
	none	0.005	8.59	89	88
	none	0.01	9.60	76	89
	0.8	0.001	7.15	39	8.0
	0.8	0.002	7.36	28	12
	0.8	0.005	8.56	ns	ns

Continued

Table 4.2.10. Continued.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
S. enteritidis	none	none	7.18	100	82
	0.8	none	6.99	100	90
	none	0.002	7.50	100	100
	none	0.005	8.24	97	90
	none	0.01	9.36	100	98
	0.8	0.002	7.24	85	61
	0.8	0.005	8.08	89	68
	0.8	0.01	9.09	59	10
C. jejuni	none	none	7.21	100	66
	0.8	none	6.53	78	68
	none	0.005	8.34	71	66
	none	0.01	9.48	47	42
	0.8	0.005	7.38	37	37
	0.8	0.01	9.42	ns	ns

ns, no survivors detected; cell kill > 99.7 %.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water (all containing 50 % v/v serum).

4.2.2.7.4. Effect of organic load on killing of Gram +ve cells by TSP.

The Gram +ve test organisms were more resistant than the Gram -ve organisms to TSP killing; however, serum also promoted survival of *L. monocytogenes* and *Staph. aureus* at both 37 °C and 4 °C (Tables 4.2.11 and 4.2.12). For example, at 37 °C in the absence of serum, 0.10 M TSP gave high kills (> 99.7 %) of both *L. monocytogenes* and *Staph. aureus* (Table 4.2.7), whereas in the presence of serum kills were reduced to 72 % and 95 %, respectively (Table 4.2.11). The pH values of 0.10 M TSP solutions, with and without serum respectively, were approximately 11.8 and 12.5 (c.f. Tables 4.2.7 and 4.2.8 with Tables 4.2.11 and 4.2.12). In the presence of serum, killing by TSP alone was more effective at 37 than at 4 °C, particularly for *Staph. aureus*. A similar effect was seen in the absence of serum (see Tables 4.2.7 and 4.2.8).

4.2.2.7.5. Effect of organic load on killing by osmotic shock and/or lysozyme.

In the absence of serum, osmotic shock treatment (0.8 M NaCl) at 37 or 4 °C, was generally ineffective against the Gram +ve organisms (Tables 4.2.7 and 4.2.8) though there was a relatively small reduction (36 %) in the viability of *Staph. aureus* at 37 °C. Similar results were obtained in the presence of serum though at 37 °C, cell kills of *Staph. aureus* were reduced to 16 % (Table 4.2.11). Similarly, the presence of serum did not alter the susceptibility of the test species to lysozyme. Thus, lysozyme treatment, in the absence of osmotic shock or TSP, had no effect on the cell viability of *Staph. aureus*, though cell kills for *L. monocytogenes* were high (>99.7 and 60 % at 37 and 4 °C respectively; Tables 4.2.11 and 4.2.12) and comparable to those without serum.

For the Gram +ve organisms, cell kills were not enhanced by combining osmotic shock and lysozyme treatments at either 37 or 4 °C. In the presence and absence of serum, the low kill (< 20 %) of *Staph. aureus* at 37 °C was similar to that for osmotic shock treatment alone and the higher kills (98 % and 77 %, at 37 and 4 °C respectively) of *L. monocytogenes* appeared attributable solely to the effect of lysozyme. It appeared

possible that 'osmotic shock' treatment may even have increased resistance of *L. monocytogenes* to lysozyme, possibly as a consequence of the 'carry over' of NaCl to the lysozyme solution. Cell kill at 37 °C fell from > 99.7 % to 98 % when cells were previously incubated in 0.8 M NaCl.

4.2.2.7.6. Effect of organic load on killing of Gram +ve cells by osmotic shock/lysozyme combined treatments with TSP.

TSP plus osmotic shock treatments, in the presence of serum, followed by exposure to lysozyme was effective in killing *L. monocytogenes*. Within the system there was a clearly synergistic interaction between the effect of TSP and osmotic shock, and between the combined treatments and exposure to lysozyme (Table 4.2.11 and 4.2.12). However, in comparison to data in the absence of serum, higher TSP concentrations (0.05 M to achieve 95 and >99 % kills at 4 and 37 °C, respectively) were required to give comparable kills. The killing of *Staph. aureus* cells using the combined treatment was similarly affected by the presence of serum. At 37 °C, high cell kills (≥ 99 %) could be obtained, but only by increasing the TSP concentration 10-fold (from 0.01 to 0.10 M; c.f. Tables 4.2.7 and 4.2.11). At 4 °C, cells were much more resistant. In the absence of serum, using 0.1 M TSP, the cell kill was only 87 % (Table 4.2.8). In the presence of serum, maximum kills were < 20 %, even at 0.15 M TSP. In experiments conducted in the presence of serum, it was evident that even following osmotic shock and TSP treatments, *Staph. aureus* was resistant to lysozyme.

Table 4.2.11. The effect of TSP, osmotic shock and lysozyme on the survival of Gram +ve bacteria at 37 °C in the presence of 50 % serum.

Cells were diluted by a factor of 1:100 into TSP + serum, NaCl + serum, TSP + NaCl + serum or deionised water + serum at 37 °C. All suspensions were incubated for 10 min. Cells were then diluted by a factor of 1:100 into deionised water or lysozyme solutions at 37 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5×10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
L .mono-cytogenes	none	none	7.12	100	ns
	0.8	none	6.99	100	1.3
	none	0.010	9.48	98	2.6
	none	0.050	11.44	52	ns
	none	0.100	12.01	28	ns
	0.8	0.010	9.42	90	2.6
	0.8	0.05	11.21	22	ns
	0.8	0.10	11.60	ns	ns
Staph. aureus	none	none	7.26	100	99
	0.8	none	7.07	84	85
	none	0.010	9.57	92	80
	none	0.050	11.50	89	82
	none	0.100	11.89	6.7	8.7
	none	0.150	12.11	ns	ns
	0.8	0.010	9.14	88	89
	0.8	0.050	10.98	92	ns
	0.8	0.100	11.48	1.2	2.3
	0.8	0.150	11.72	ns	ns

ns, no survivors detected; cell kill > 99.7 %.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water (all containing 50 % v/v serum).

Table 4.2.12. The combined effect of TSP, osmotic shock and lysozyme on the survival of Gram +ve bacteria at 4 °C in the presence of 50 % v/v serum.

Cells were diluted by a factor of 1:100 into TSP + serum, NaCl + serum, TSP + NaCl + serum or deionised water + serum at 4 °C. All suspensions were incubated for 10 min. Cells were then diluted by a factor of 1:100 into deionised water or lysozyme solutions at 37 °C and incubated for 30 min. The initial cell concentrations in the first set of treatment solutions were approximately 2.5 x 10^7 cfu ml^{-1}.

Organism	NaCl (M)	TSP (M)	pH*	% cell survival	
				- lysozyme	+ lysozyme
L. mono-cytogenes	none	none	7.15	100	37
	0.8	none	6.90	100	23
	none	0.010	9.71	100	54
	none	0.050	11.38	74	4.7
	none	0.100	11.71	12	1.9
	0.8	0.010	9.36	74	27
	0.8	0.050	10.94	2.0	ns
	0.8	0.10	11.71	2.0	ns
Staph. aureus	none	none	7.44	100	100
	0.8	none	7.18	100	100
	none	0.01	9.67	100	100
	none	0.05	11.53	100	100
	none	0.10	11.82	100	100
	none	0.15	12.10	100	89
	0.8	0.01	9.39	100	100
	0.8	0.05	11.07	100	100
	0.8	0.10	11.51	100	100
	0.8	0.15	11.69	95	82

ns, no survivors detected; cell kill > 99.7%

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water (all containing 50 % v/v serum).

4.2.3. DISCUSSION

The experimental work carried out in this section of the thesis (Section 4.2) was to investigate the hypothesis that low concentrations of TSP might promote susceptibility of Gram -ve bacteria to lysozyme and/or osmotic shock procedures. TSP is known to kill Gram -ve organisms; however, high concentrations (approximately 10 %) are required to effectively decontaminate the surface of foods (Kim and Slavik, 1994a, 1994b).

The possible mechanisms of TSP killing and food decontamination have been discussed in Section 4.1. It is plausible that a role of TSP is as a surfactant and that it may initially disrupt the outer membrane of the Gram -ve cell wall, so explaining its greater effectiveness against Gram -ve, rather than Gram +ve bacteria. The results obtained in Section 4.2.2.1, for a range of test organisms, confirmed that suspended cells of Gram -ve species were markedly more sensitive to TSP than cells of Gram +ve species. These experiments also identified the range of TSP concentrations which produced little or no killing of the test species, thus , enabling further experiments to be conducted in which synergistic relationships between low TSP concentrations and exposure to lysozyme and/or osmotic shock could be compared. For suspended cells of *E. coli*, *C. jejuni*, *P. fluorescens* and *S. enteritidis*, there was a marked synergy between TSP and lysozyme treatments. Presumably, at low concentrations, TSP was able to sufficiently disrupt the outer membrane of the cell wall of these bacteria, to enable lysozyme to reach the peptidoglycan layer. Susceptibility to TSP-lysozyme killing was greater at 37 °C than at 4 °C, particularly for *C. jejuni* which also shows increased resistance to TSP (Somers *et al.*, 1994) and other cell killing procedures at low temperatures (Clavero *et al.*, 1994). TSP treatments have been generally reported to be more effective at higher temperatures (Dickson *et al*, 1994; Kim *et al*, 1994). This is presumably due to an increase in outer membrane and cell-membrane fluidity, facilitating the disruptive effect of TSP.

There was also a synergistic effect on the killing of Gram -ve species when TSP treatment was combined with osmotic shock. However, in the data shown in Tables 4.2.4 and 4.2.5, the extent of the interaction was variable For *P. fluorescens* at 37 °C osmotic shock and treatment with 0.001 M TSP had no effect when applied separately, but when combined, produced a kill of > 99 %. For *S. enteritidis* at 37 °C, osmotic shock and 0.001 M TSP gave kills of 14 % and 9 % respectively, but the combined treatment gave only a 26 % kill. Such variability might reflect different optimal values for osmotic shock, which was not investigated. The interaction between osmotic shock and TSP may again be due to TSP disruption of the outer-membrane of the cell wall, increasing the susceptibility of cells to osmotic lysis when subsequently diluted in deionised water. It is also possible that during hyper-osmotic shock (i.e. during incubation in NaCl plus TSP), TSP might more easily penetrate the cell wall and gain greater access to the cell membrane. Disruption of the cell membrane would lead to cell death.

In the combined TSP plus osmotic shock and lysozyme treatment, it was clear that under the conditions used, all three components of the treatment were able to contribute to cell killing. However, it was noted that at 4 °C, the contribution of lysozyme was less important, particularly for *C. jejuni*. Combined treatments with osmotic shock and lysozyme enabled the TSP concentration required to give high kills (> 99 %) of Gram -ve test organisms, at 4 and 37 °C, to be reduced to 0.001 or 0.002 M (except for *C. jejuni*, 0.005 M at 4 °C). An important aspect of the combined treatment was that it was also effective to some extent against Gram +ve organisms. *Listeria monocytogenes* appeared most sensitive to the lysozyme component of the treatment but kills were also enhanced by a low concentration of TSP and osmotic shock. In contrast, *Staph. aureus* was little affected by lysozyme under the conditions used (30 min exposure; 20 $\mu g\ ml^{-1}$). At 0.005 M TSP, the kill of *Staph. aureus* could also be increased to a high level (99.7 %).

When the test organisms were exposed to TSP/osmotic shock/lysozyme treatments in the presence of serum (50 % v/v), a significant decrease in cell killing was observed where low TSP concentrations were used. There are many previous reports which show that the presence of organic matter may reduce the bactericidal efficacy of disinfectant procedures (e.g. Lillard, 1980; Mullerat *et al.*, 1995). However, the protective effects of organic load on bacterial survival, in the TSP/osmotic shock/lysozyme combined treatment, could be overcome by increasing the TSP concentration. Importantly, this increase in TSP concentration did not lead to a corresponding increase in the pH of the reaction mixtures due to the buffering influence of the serum.

In conclusion, the results suggest that TSP does enhance the susceptibility of suspended cells of Gram -ve species to lysozyme and osmotic shock. The TSP concentrations required to give essentially total kills ($\geq$ 99.7 % reduction in viability, no survivors detected) in the combined procedure, were $\leq$ 0.005 M, at both 4 and 37 °C. Similar concentrations also enabled effective killing of *L. monocytogenes* and to a lesser extent *Staph. aureus* (37 °C only). The low TSP concentrations required for the combined treatment suggest that it might be used as an alternative to high concentration (high pH) TSP-killing in the decontamination of foodstuffs. The pH of treatment fluids containing 0.005 M TSP was typically $\leq$ 10.5. Thus, experiments were conducted to test the system developed on organisms attached to food and to food - contact surfaces. It was recognised that the presence of organic matter (the food substance or food material contaminating food contact surfaces) might inactivate or otherwise reduce the effectiveness of TSP, as observed for serum.

Therefore, in further experiments, it might be necessary to raise the concentration of TSP above 0.005 M. In the presence of 50 % serum, 0.01 M TSP was necessary to give high kills of all of the Gram -ve test organisms and *L. monocytogenes*. However, the pH of 0.01 M TSP solutions were reduced to < 10 by the presence of serum.

4.3. The effect of combined trisodium phosphate, osmotic shock and lysozyme treatment on microorganisms attached to food and food-contact surfaces.

4.3.1. INTRODUCTION

Chatzopoulou (1991) previously demonstrated that combined killing procedures involving lysozyme and osmotic shock at both high (20 to 37 °C) and low temperatures (0 to 10 °C), reduced the microbial populations on red meat and poultry skin artificially contaminated with *S. typhimurium*. A reduction of up to approximately 90 % of the population was observed for both high and low temperature procedures; however, cell kills were not as high as those observed for cells in suspension. Large reductions in the number of organisms recovered in the washing solutions were also observed.

Low concentrations of TSP (0.001 M to 0.005 M) in combination with modified forms of the osmotic shock and/or lysozyme treatments developed by Chatzopoulou (1991), gave high kills (no survivors detected) for suspended cells of Gram -ve test organisms (*C. jejuni, P. fluorescens, S. enteritidis* and *E. coli*) and *L. monocytogenes,* at both 4 and 37 °C (Section 4.2). The treatment was also partially effective, at 37 °C, against *Staph. aureus*.

The efficiency of the TSP combined treatments in killing suspended cells suggested that they might also be successful in killing pathogenic and spoilage organisms on foods. This was investigated using chicken as the test food. Chicken carcasses carry potentially large numbers of both spoilage organisms and human pathogens, mainly *Campylobacter* and *Salmonella* spp. There is also concern that avian pathogenic *E. coli* (APEC) strains, which are toxin producing, may also be transmissible to man. Fresh chicken thigh portions were purchased from a retail outlet and inoculated with *S.*

enteritidis or *E. coli.* These organisms were selected as being representative of commonly encountered Gram -ve food-borne pathogens. Artificially contaminated experimental samples inoculated with up to 10^8 cfu g^{-1} were used in preference to naturally contaminated samples, to avoid the technical difficulties associated with detecting low, but significant levels, of foodborne organisms; under normal circumstances it is unusual for counts of, for example, salmonellae to exceed 10^3 cfu g^{-1} (Bremner and Johnston, 1996). The experimental procedure adopted was to wash inoculated chicken skin samples by immersion in solutions of TSP and/or NaCl and then to rinse them in a dilute lysozyme solution or water. Following treatment, bacterial counts on the chicken samples were determined by stomaching (as recommended by the British Poultry Meat Association Ltd.; Parry *et al.*, 1982). Alternatively, in experiments using inoculated, but previously irradiated chicken skin, bacteria surviving treatment and remaining on the skin surface were detected by the *in situ* NBT detection method described in Section 3.1. In experiments using chicken, bacterial counts were also determined for the treatment solutions; the efficiency with which organisms suspended in treatment solutions are killed is important in relation to cross-contamination of carcasses in poultry processing environments.

It was anticipated that there would be a decrease in cell killing for bacteria attached to chicken skin, compared to kills in experiments using suspended cells, because it is widely reported that suspended cells are generally more susceptible to bactericidal treatments (Costerton *et al.*, 1995). Chatzopoulou (1991) also found in her experiments that kills on the chicken surface were disappointingly low compared to data for cells in suspension. Therefore, the experimental killing procedures developed here were also

tested on contrasting food types (prawns and lettuce) and inert food preparation surfaces (stainless steel and ceramic). These experiments were considered necessary in order to determine whether the chicken skin surface had a particularly protective effect on survival, due to the presence of surface crevices or protective substances (e.g. fats). However, it is also possible that the killing procedures developed might find application in the decontamination of food preparation or other surfaces, or in the treatment of salad vegetables or seafoods.

4.3.2. RESULTS

4.3.2.1. The effect of osmotic shock, lysozyme and TSP treatments on the survival of bacteria attached to chicken skin: methodology.

The numbers of endogenous bacteria present on chicken skin samples (excised from commercially prepared chicken thigh portions), was typically $\geq 10^3$ cfu g^{-1}. Thus, to reduce confusion of inoculated test bacteria with endogenous bacteria, the surface of chicken skin samples was inoculated with 20 µl of undiluted early stationary phase cultures of the test bacteria (*E. coli* or *S. enteritidis*). A drying time of 30 min was allowed. Bacterial attachment to animal tissues increases over time and Dickson (1991) recorded the highest attachment after 20 min. Alternatively, the chicken skin samples were immersed in 25 ml of early stationary phase bacterial cultures for 30 min at ambient temperature and then dried for 3 min in a stream of cold air. In both methods, the numbers of viable bacteria on skin samples was typically $\geq 10^8$ cfu g^{-1}, i.e. much greater than the numbers of endogenous bacteria. Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water. After incubation for 10 min at ambient temperature (20-25 °C) the samples were shaken to remove excess fluid, then transferred to deionised water or deionised water containing 100 µg ml^{-1} lysozyme. After incubation for 30 min, samples were stomached individually for 2 min. Serial dilutions of stomached samples, and where indicated, treatment fluids, were plated on nutrient agar.

4.3.2.2. The effect of TSP on the survival of *E. coli* attached to chicken skin.

The effect of a 10 min exposure to TSP on the survival of *E. coli* attached to chicken skin is shown in Table 4.3.1, for experiments in which chicken skin was inoculated by immersion in culture. Previously, complete killing of suspended *E. coli* was observed at 0.005 M TSP (Tables 4.2.1 and 4.2.2); however, for organisms attached to skin, higher concentrations (> 0.05 M) were required to give kills of > 90%, and total kills were not achieved even at 0.3 M TSP (11 %, w/v). This was not simply due to an effect of the chicken skin on the pH of TSP solutions, since, when attached to skin survival rates of up to almost 90 % were seen at pH values >12.

Interestingly, and in agreement with previous results showing the susceptibility of suspended Gram -ve cells to TSP (Tables 4.2.9 and 4.2.10), *E. coli* was not detected in TSP wash solutions, where the concentration of TSP was ≥0.01 M TSP (Table 4.3.2). However, where water was used to wash TSP-treated skin, *E. coli* was recovered (6.0 x 10^3 cfu g^{-1} chicken skin) even after the skin had been exposed to 0.1 M TSP. The recovery of skin attached inoculated organisms in this case was 8 %, compared to an equivalent treatment without TSP. Therefore, it would appear that adhered organisms not killed by TSP, may be detached and subsequently recovered in water rinses.

The killing of *E. coli* on the skin surface by TSP was rapid (Table 4.3.3). At both high (0.4 M) and relatively low (0.02 M) concentrations, cell kills close to the maximum values for these concentrations (approximately 95 and 99 %, respectively) were obtained following a 1 min exposure.

The substantial protective effect of chicken skin on the survival of bacteria subjected to chemical stress, has previously been noted by Lillard (1988). The results presented here support his general conclusion and are also in agreement with previous work which has consistently demonstrated the necessity for high TSP concentrations to substantially reduce populations of *S. typhimurium*, *Campylobacter* species, *E. coli and L. monocytogenes* on poultry, beef and pork (Kim and Slavik, 1994b; Dickson *et al.*, 1994; Kim *et al.*, 1994; Slavik *et al.*, 1994; Morris *et al.*, 1997). The possibility of combining TSP treatment with exposure to osmotic shock and/or lysozyme, so as to reduce the TSP concentration required, is considered in the following section (Section 4.3.2.3).

Table 4.3.1. The effect of TSP on the survival of *E. coli* attached to chicken skin incubated at room temperature.

Chicken skin samples (1 g) were immersed in an early stationary phase *E. coli* culture for 3 min and then dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP solutions. After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature, samples were stomached individually for 2 min. Serial dilutions of stomached samples were plated on nutrient agar.

TSP (M)	pH*	% Survival
none	7.12	100**
0.05	12.32	89
0.10	12.46	8.0
0.15	12.74	2.0
0.20	12.77	1.5
0.30	12.92	0.50

*pH of TSP solution after incubation with chicken skin for 10 min.

** 9.5×10^8 cfu g^{-1}.

Table 4.3.2. The recovery of *E coli* attached to chicken skin in TSP and water wash solutions.

Inoculated chicken skin samples (1 g) were incubated in TSP solutions (10 ml) for 10 min and then transferred to deionised water (10 ml) for 30 min. Both treatments were carried out on a rotary shaker (100 rpm) at ambient temperature (22 °C). The number of organisms recovered on skin varied between 3×10^8 cfu g^{-1} (control, TSP absent) and 2×10^5 cfu g^{-1} at the highest TSP concentrations used. Wash solutions were plated on nutrient agar.

TSP (M)	pH of TSP solution*	pH of water rinse solution*	Recovery from TSP solution (cfu (g skin)$^{-1}$)	Recovery from rinse water (cfu (g skin)$^{-1}$)
none	6.71	6.92	6.5×10^6	1.3×10^3
0.002	7.36	6.93	5.6×10^5	1.0×10^4
0.005	10.21	6.97	1.0×10^3	1.0×10^4
0.01	11.08	7.02	ns	1.25×10^4
0.02	11.56	6.85	ns	1.6×10^3
0.05	11.88	7.10	ns	4.2×10^3
0.10	12.12	7.13	ns	6.0×10^3

* After being used to treat chicken skin.

ns, no survivors detected; recovery $<10^2$ g^{-1} chicken skin sample.

Table 4.3.3. The effect of TSP treatment time on the % survival of *E. coli* attached to chicken skin.

Chicken skin samples (1 g) were inoculated with 20 µl of *E. coli* culture and dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP solutions. After incubation for between 0 and 30 min on a rotary shaker (100 rpm) at ambient temperature, samples were stomached individually for 2 min. Serial dilutions of stomached samples were plated on nutrient agar.

TSP (M)	% Survival at various times (min)				
	0	1	2	10	30
0.02	100	6	4	13	10
0.40	100	2	0.5	2	0.2

4.3.2.3. The effect of TSP, osmotic shock and lysozyme treatments on the survival of *E. coli* attached to food.

4.3.2.3.1. The effect of high TSP concentrations (≥ 0.01 M), osmotic shock and lysozyme treatments on the survival of *E. coli* attached to chicken skin.

In initial experiments, chicken skin was inoculated by immersion in *E. coli* culture and shaken for 10 min at room temperature in either TSP solution or TSP containing 0.8 M NaCl. The skin was then removed from TSP solution and shaken for a further 30 min at room temperature in either deionised water or deionised water containing lysozyme. The results (Table 4.3.4) show a modest kill (up to 80 % in replicate experiments) by osmotic shock plus lysozyme treatment. This was not significantly increased by combined treatment with TSP at concentrations of 0.01 and 0.05 M. Kills of approximately 99 % were obtained by increasing the TSP concentration to 0.1 M and above; however, at these high TSP concentrations, there was no apparent synergistic effect between TSP and osmotic shock and/or lysozyme treatment. A similar lack of a synergistic interaction was also evident at these TSP concentrations (0.1 and 0.2 M), in experiments conducted at 4 °C (Table 4.3.5).

It was considered possible that synergistic interactions with lysozyme, at the TSP concentrations tested, were inhibited by high pH. The pH of TSP/NaCl solutions ranged from approximately 9.5 (0.01 M TSP) to >12 (0.2 M TSP) (Tables 4.3.4 and 4.3.5). A substantial carryover of TSP to lysozyme solutions was evident, since, for example, following treatment with 0.2 M TSP, the pH of the lysozyme solution was

above 11 (Table 4.3.4 (B)). Further control experiments revealed that carryover was of the order of 0.1 to 0.3 g of TSP solution per g chicken skin, even though skin samples removed from TSP solutions were shaken prior to immersion in lysozyme or deionised water.

The kills of chicken skin-attached bacteria by the combined TSP, osmotic shock and lysozyme treatment were lower than those anticipated (Section 4.2) (at equivalent TSP concentrations) from killing experiments of suspended organisms in the presence of a high organic load. The absence of a synergic effect at the higher TSP concentrations used was also unexpected. It was possible that the chicken skin surface specifically reduced the success of the combined treatment, therefore experiments were repeated using prawns (Section 4.3.2.3.2), food contact surfaces (Section 4.3.2.4) and lettuce leaves (Section 4.3.2.5).

Table 4.3.4. The effect of TSP, osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at ambient temperature. **Table A** shows the % survival of *E. coli* attached to chicken skin after treatment with TSP, osmotic shock and lysozyme. **Table B** shows the pH values for processing solutions following initial treatments in the presence or absence of 0.20 M TSP.

Chicken skin samples (1 g) were immersed in an early stationary phase *E. coli* culture for 3 min and dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature (22 °C) the samples were transferred to deionised water or deionised water containing 100 μg ml^{-1} lysozyme (10 ml). After incubation for 10 min on a rotary shaker (100 rpm), samples were stomached individually for 2 min. Serial dilutions of stomached samples, and where indicated, treatment fluids, were plated on nutrient agar.

A

NaCl (M)	TSP (M)	pH of initial treatment solution (after use)*	% Cell Survival	
			-Lysozyme	+ Lysozyme
none	none	6.64	100	98
0.8	none	6.39	86	20
none	0.01	9.82	100	84
none	0.05	11.69	93	53
none	0.10	11.99	0.1	1.8
none	0.2	12.34	0.8	0.4
0.8	0.01	9.24	45	45
0.8	0.05	11.04	53	39
0.8	0.10	11.92	0.2	0.1
0.8	0.20	12.27	0.7	0.4

Continued

Table 4.3.4. Continued.

B

NaCl (M)	TSP (M)	pH of final treatment solution (after use)**	
		-Lysozyme	+Lysozyme
none	none	6.62	6.48
0.8	none	6.50	6.52
none	0.20	11.67	11.68
0.8	0.20	11.56	11.56

*initial treatment solutions were: TSP, TSP + NaCl, or water.

**final treatment solutions were: lysozyme solution or water.

Table 4.3.5. The effect of TSP, osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at 4 °C.

Chicken skin samples (1 g) were immersed in an early stationary phase *E. coli* culture for 3 min and dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at 4 °C, the samples were transferred to deionised water or deionised water containing 100 μg ml^{-1} lysozyme (10 ml) at 4 °C. After incubation for a further 10 min on a rotary shaker (100 rpm) at 4 °C, samples were stomached individually for 2 min. Serial dilutions of stomached samples, and where indicated, treatment fluids, were plated on nutrient agar.

NaCl (M)	TSP (M)	pH*	% Cell survival	
			-Lysozyme	+Lysozyme
none	none	6.73	100	91
0.8	none	6.36	100	41
none	0.10	12.22	1.2	1.2
none	0.20	12.46	0.10	0.15
0.8	0.10	11.95	5.6	3.9
0.8	0.20	12.30	6.4	1.14

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water after use.

4.3.2.3.2. The effect of high TSP concentrations (≥ 0.01 M), osmotic shock and lysozyme treatments on the survival of *E. coli* attached to prawns.

Experiments were conducted to test the survival of *E. coli* on prawns which had been inoculated by immersion in an early stationary phase culture for 3 min, followed by drying in air. In this experimental system, cell kills of 98.0 % were obtained using 0.1 M TSP and 99.8 % using 0.1 M TSP in combination with osmotic shock. Cell kills in the absence of TSP or with 0.01 M TSP were less than 60 % (Table 4.3.7). There was little evidence of any synergic effect of TSP/osmotic shock treatment with exposure to lysozyme, at the TSP concentrations used. Thus, the results of these experiments with prawns were similar to those using organisms attached to chicken skin. They confirmed that high concentrations of TSP, in combination treatments, did not increase the killing of cells attached to these types of food.

Table 4.3.6. The effect of TSP, osmotic shock and lysozyme on the survival of *E. coli* attached to prawns.

Prawns (0.5 g) were immersed in an early stationary phase *E. coli* culture for 3 min and dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature (22 °C) the samples were transferred to deionised water or deionised water containing 100 μg ml^{-1} lysozyme (10 ml). After incubation for 10 min on a rotary shaker (100 rpm), samples were stomached individually for 2 min. Serial dilutions of stomached samples were plated on nutrient agar.

NaCl (M)	TSP (M)	pH*	% Cell survival	
			-Lysozyme	+Lysozyme
none	none	7.25	100	62
0.8	none	6.66	47	40
none	0.01	9.62	78	78
0.8	0.01	9.34	75	73
none	none	7.16	100	30
0.8	none	6.66	80	67
none	0.10	12.15	2.1	2.4
0.8	0.10	11.77	0.40	0.20

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water at the end of experiments.

4.3.2.4. The effect of osmotic shock, lysozyme and TSP treatments on the survival of bacteria attached to food contact surfaces.

The experiments conducted using food contact surfaces (ceramic and stainless steel) had two main objectives. One was to further evaluate the role of the surface type in the resistance of attached bacteria to combined killing treatments. The other was to assess the potential of combined treatments in the decontamination of such surfaces.

The test surfaces were inoculated with *S. enteritidis* suspended in 10 % serum or 5 % NaCl and dried for 3 h in air at 37 °C. Inoculated surfaces (ceramic and stainless steel) were immersed in TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water and, after incubation for 2 min, were transferred to deionised water or deionised water containing lysozyme. Attached bacteria on the test surfaces were then detected by overlaying with molten nutrient agar and allowing colony growth. Colonies were visualised using NBT.

In control experiments, water washes greatly reduced the numbers of *S. enteritidis* attached to ceramic and stainless steel surfaces. The high levels of reduction (99.5 and 99.8 %, respectively) were similar to those obtained in previous experiments (Section 3.3). Treatment of the contaminated surfaces with TSP alone, resulted in further reductions in the counts of attached bacteria. On stainless steel, the total reduction in counts was > 99.99 % (no survivors detected) and on ceramic 99.8 % (Table 4.3.7). On the ceramic surface, both osmotic shock and lysozyme treatment reduced the numbers

of bacteria detected, and following the TSP/osmotic shock/lysozyme combined treatment, no survivors were detected.

The results from this series of experiments show that surface type may significantly influence the success of decontamination treatments. The greater effectiveness of the decontamination procedures used on the stainless steel surface was observed in replicate experiments. However, on both food contact surfaces, the effectiveness of the procedure was much greater than on the chicken skin or prawn surface (Sections 4.3.2.3.1 and 4.3.2.3.2).

Table 4.3.7. The effect of TSP, osmotic shock and lysozyme on the survival of *S. enteritidis* dried onto stainless steel and ceramic samples.

The test surfaces (9 x 9 cm^2) were inoculated with 10 x 20 µl of *S. enteritidis* culture and dried for 3 h in air at 37 °C. They were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (100 ml) and incubated for 2 min on a rotary shaker (100 rpm) at ambient temperature (22 °C). They were then transferred to deionised water or deionised water containing 100 µg ml^{-1} lysozyme (100 ml). After incubation for a further 2 min on a rotary shaker (100 rpm), the surfaces were shaken to remove the treatment solutions and overlaid with nutrient agar. Colonies developing on the test surfaces were visualised with NBT after overnight incubation at 37 °C.

Surface	NaCl (M)	TSP (M)	pH*	Organisms recovered (% of organisms recovered from unwashed surface)	
				-Lysozyme	+Lysozyme
Stainless steel	none	none	6.92	0.16	0.085
Stainless steel	none	0.005	10.49	ns	ns
Stainless steel	0.8	0.005	10.23	ns	ns
ceramic	none	none	6.97	0.55	0.08
ceramic	none	0.005	10.22	0.024	0.018
ceramic	0.8	0.005	9.98	0.0089	ns

ns, no survivors detected.

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water, at the end of the experiment.

4.3.2.5. The effect of TSP, osmotic shock and lysozyme treatments on the survival of *E. coli* and *S. enteritidis* on lettuce leaf sections.

TSP and combined TSP/osmotic shock/lysozyme treatments appeared much more effective against organisms on food surfaces (ceramic, stainless steel) than against organisms attached to chicken skin and prawns. It appeared possible that the surfaces of chicken and prawns might occlude organisms within surface crevices or pores, thereby providing protection against the decontamination treatments. The effect of surface type on the efficacy of the treatments was therefore investigated using lettuce leaf sections. Lettuce leaf sections were selected as being representative of an alternative type of food with a less heterogeneous surface. The results of experiments using lettuce would help distinguish whether treatments were less effective on surfaces of organic material or whether the key factor was the topography of the surface. Also, if the treatments were successful on lettuce, it is possible they might find an application in the decontamination of salad and vegetable materials for processed foods.

In experiments on lettuce using *E. coli*, kills of attached bacteria by TSP (0.002 to 0.05 M) treatment alone were up to 77 % (Table 4.3.8). However, there was a marked synergy between TSP and lysozyme treatments (kills up to > 95 %), particularly at the lower TSP concentrations (<0.01 M) used. At higher concentrations the effect of lysozyme was less marked. TSP may inhibit lysozyme activity at high concentrations either directly or via effects on pH. However, the pH of lysozyme solutions after the addition of TSP-treated lettuce was in all cases approximately 7 (Table 4.3.8 (B)). Therefore, any effect due to pH would have to be within a surface film, which had not

been quickly mixed with the bulk lysozyme solution. Slow mixing might allow stabilisation of the outer membrane of the *E. coli* cells before the inner regions of the cell wall were effectively exposed to active enzyme. The results on lettuce leaves (Table 4.3.8 (A)) also showed a synergy between TSP and osmotic shock treatments, especially at 0.01 M TSP (the highest concentration tested) where cell kill was >99 %. A single experiment was also conducted using *S. enteritidis* attached to lettuce. Results (Table 4.3.9) were qualitatively similar to those for *E. coli*, though cell kills were generally somewhat lower. The pH values of the treatment solutions used in experiments with lettuce are given in Tables 4.3.8 (B) & 4.3.9 (B).

The successful decontamination of both lettuce leaf sections and food contact surfaces with TSP/osmotic shock/lysozyme combined treatments, demonstrated that attached organisms were susceptible. Therefore, it appears that surface attachment *per se* may have a relatively small effect on bacterial resistance to the killing treatments developed. The lower kills observed for bacteria on the surface of chicken and prawns might therefore be strongly influenced by surface topography. However, experiments conducted with lettuce leaf sections showed that other factors could be significant. The carry-over of TSP into lysozyme solutions, increasing the pH at the cell surface and reducing lysozyme activity, might be particularly important at TSP concentrations of >0.01 M. Additionally, proteins, fatty material and serous fluids leaching out of the excised chicken skin, and particularly the underside of the chicken skin, might adversely influence pH during the TSP treatment.

Table 4.3.8. The effect of TSP, osmotic shock and lysozyme on the survival of *E. coli* attached to lettuce leaf at ambient temperature. Table A shows the number (%) of *E. coli* attached to chicken skin after treatment with TSP, osmotic shock and/or lysozyme. **Table B** shows the pH values for processing solutions following initial treatments in the presence or absence of TSP (0.002 -0.10 M).

Lettuce leaf (circular sections; 3 cm diameter) were inoculated with an early stationary phase *E. coli* culture and dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature (22 °C) the samples were transferred to deionised water or deionised water containing 100 $\mu g\ ml^{-1}$ lysozyme (10 ml). After incubation for 10 min on the rotary shaker (100 rpm), samples were stomached individually for 2 min. Serial dilutions of stomached samples, and where indicated, treatment fluids, were plated on nutrient agar.

A

NaCl (M)	TSP (M)	% Cell Survival	
		- lysozyme	+ lysozyme
none	none	100	42
0.8	none	22	5.6
none	0.002	85	ns
none	0.005	56	4.9
none	0.01	66	53
none	0.02	77	17
none	0.05	23	5.2
0.8	0.002	52	29
0.8	0.005	29	3.4
0.8	0.01	0.58	0.39

ns, no survivors detected; cell kill > 99.7 %.

Continued

Table 4.3.8. Continued.

B

NaCl (M)	TSP (M)	pH of final treatment solutions*	
		- Lysozyme	+ Lysozyme
none	none	7.24	6.95
0.8	none	7.00	6.89
none	0.002	7.07	6.81
none	0.005	7.03	6.94
none	0.01	7.01	6.83
none	0.02	6.90	6.88
none	0.05	6.91	6.96
0.8	0.002	6.94	6.84
0.8	0.005	6.91	6.86
0.8	0.01	7.02	6.97

*final treatment solutions were lysozyme solution or water; pH was measured at the end of experiments.

Table 4.3.9. The effect of TSP, osmotic shock and lysozyme on the survival of *S. enteritidis* attached to lettuce leaf at ambient temperature. Table A shows the number (%) of *S. enteritidis* attached to lettuce after treatment with TSP, osmotic shock and/or lysozyme. **Table B** shows the pH values for processing solutions following initial treatments in the presence or absence of TSP (0.001 - 0.10 M).

The experiment was conducted as described in Table 4.3.8, except that the test organism used was *S. enteritidis*.

A

NaCl (M)	TSP (M)	% Cell Survival	
		- Lysozyme	+ Lysozyme
none	none	100	74
0.8	none	27	20
none	0.001	100	16
none	0.002	95	74
none	0.005	68	56
none	0.01	98	44
0.8	0.001	64	18
0.8	0.002	24	23
0.8	0.005	19	9.9
0.8	0.01	31	13

Continued

Table 4.3.9. Continued.

B

NaCl (M)	TSP (M)	pH of final treatment solutions*	
		-Lysozyme	+ Lysozyme
none	none	6.99	7.01
0.8	none	6.89	6.92
none	0.001	6.95	6.85
none	0.002	6.98	7.03
none	0.005	7.03	7.03
none	0.01	7.05	7.01
0.8	0.001	6.83	6.81
0.8	0.002	7.02	6.97
0.8	0.005	6.96	7.01
0.8	0.01	7.05	7.07

*final treatment solutions were lysozyme solution or water; pH was measured at the end of the experiments.

4.3.2.6. The effect of **low** TSP concentrations (≤ 0.01 M), osmotic shock and lysozyme treatments on the survival of *E. coli* attached to chicken skin.

Previous experimental results (Sections 4.3.2.3.1 and 4.3.2.3.2) showed relatively low levels of synergy between TSP, osmotic shock and lysozyme treatments, against organisms attached to chicken skin and prawns. However, the treatments were more effective against organisms attached to impervious food contact surfaces and to lettuce. In these experiments there was some evidence that a major factor in reduced cell kills was the carry over of TSP from the initial treatment solution (TSP or TSP plus NaCl) to the lysozyme solution. Possibly, surface attached organisms were within a film of TSP solution which was not instantly dispersed upon transfer to the lysozyme solution.

Therefore, experiments were repeated for chicken skin at ambient temperature using lower TSP concentrations (0.002 to 0.01 M). Additionally, in these and subsequent experiments, the skin surface was directly inoculated with test organism rather than being immersed in culture (as in some of the previous experiments). The reason for adopting this method was to minimise the access of organisms to the underside of skin samples, where they might be more easily occluded by fatty material. Skin samples (1 g) were inoculated with approximately 4×10^6 cfu of test organism. The number of surviving attached organisms after a 30 min drying period, was approximately 2×10^4 cfu.

Results (Table 4.3.10) show that, for *E. coli* cells dried on the outer surface of chicken skin, there was synergy between TSP/lysozyme, TSP/osmotic shock and TSP/osmotic

shock/lysozyme treatments at low TSP concentrations. The maximum cell kill (TSP/osmotic shock/lysozyme combined treatment), observed using 0.002M TSP (pH approximately 9), was 97 %, i.e. comparable to that for cells inoculated onto the skin surface and treated for 10 min with 0.4 M TSP (pH 12.5) only (Table 4.3.3). Thus, this experiment showed that high cell kills could be achieved in the combined treatment at TSP concentrations well below those required for killing by TSP alone, but the TSP concentration needed for maximal kill was relatively, very low. This again suggests that carry over of TSP to the lysozyme solution reduces the effectiveness of the combined treatment. In experiments using ≤ 0.01 M TSP, the pH of lysozyme treatment solutions, after experiments, was in all cases ≤ 7.

However, although kills of up to 97 % were observed for *E. coli* cells attached to skin, it was clear that attached cells were more resistant or to some extent protected from the killing treatments used. Thus, for suspended cells, no survivors were detected when *E. coli* cells were subjected to the combined TSP/osmotic shock/lysozyme treatment (0.002 M TSP); whereas for attached cells subjected to the same treatment, survival was 3 %.

The kills of *E. coli* (up to 97 %; 1.99 Log_{10} reduction) on chicken skin treated with low concentrations of TSP (0.002 - 0.01 M; 0.08 - 0.4 % w/v) in the combined treatments, were comparable to kills in published work using TSP treatment only (8 -12 % w/v; 0.2 - 0.3 M). For example, Lillard (1994) showed that treating chicken skin with 10 % TSP caused a 2 Log_{10} reduction in viable counts of *S. typhimurium*. Similarly, Kim and Slavik (1994b) showed that the treatment of beef surfaces with TSP (10 % w/v) reduced the level of attached *E. coli* O157: H7 and *S. typhimurium* by 0.51 - 1.39 Log_{10} cycles. The

TSP- combined treatments developed here also improve on the bacterial killing methods developed by Chatzopoulou *et al.* (1993). These used osmotic shock combined with cold shock and exposure to lysozyme and gave kills of up to 99 % for certain Gram -ve bacteria on poultry carcasses. However, *Salmonella* appeared relatively resistant (maximum cell kill 1.1 Log_{10} reduction). Also, unless the treatment was carried out at a low temperature (< 10 °C), high kills required the presence of nutrients such as brain heart infusion. Thus, the bacterial kills achieved using the TSP combined treatments, whilst better than or comparable to kills obtained by Chatzopoulou *et al.* (1993), also offer the possibility for exploitation under a wide range of operational conditions.

Table 4.3.10. The effect of low concentrations of TSP (0.002 - 0.01 M), osmotic shock and lysozyme treatment on the survival of *E. coli* attached to chicken skin at room temperature.

Chicken skin samples (1 g) inoculated with 20 µl of *E. coli* culture were dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature (22 °C) the samples were transferred to deionised water or deionised water containing 100 µg ml^{-1} lysozyme (10 ml). After further incubation for 10 min on a rotary shaker (100 rpm), samples were stomached individually for 2 min. Serial dilutions of stomached samples were plated on nutrient agar.

NaCl (M)	TSP (M)	pH*	% Cell Survival	
			- Lysozyme	+ Lysozyme
none	none	6.93	100	44
0.8	none	6.45	32	14
none	0.002	8.83	42	14
none	0.005	10.34	50	7.4
none	0.01	11.08	44	9.7
0.8	0.002	9.16	18	3.2
0.8	0.005	9.90	25	5.0
0.8	0.01	10.58	25	7.3

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water, at the end of the experiment.

4.3.2.7. The effect of osmotic shock, lysozyme and TSP treatments on the survival of bacteria attached to food: Detection of bacterial survival by the *in situ* NBT method.

The efficacy of TSP/osmotic shock/ lysozyme treatments in reducing bacterial numbers on chicken skin was also demonstrated using the *in situ* NBT method. The skin used was excised from irradiated chicken portions to eliminate interference with the test system by endogenous bacteria. The surfaces of the previously irradiated chicken skin samples were artificially inoculated with 20 µl early stationary phase cultures of *E. coli* and allowed to dry for 30 min. Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water. Then, after incubation for 10 min at ambient temperature (22 °C), the samples were transferred to deionised water or deionised water containing 100 µg ml^{-1} lysozyme. However, instead of stomaching the samples after the washing treatments, they were overlaid with agar and the *in situ* NBT detection method (described in Section 3.1) was used to determine the number of surviving attached bacteria. A concentration of 0.005 M TSP was used in all experiments reported in this section.

Figure 4.3.1. shows, as expected, the absence of colonies on irradiated, uninoculated chicken skin. However, colonies are readily visible on irradiated skin inoculated with *E. coli* (Fig. 4.3.2). Thus, the technique would appear potentially useful for the direct enumeration of bacteria on the surface of foodstuffs. Figures 4.3.3 and 4.3.4 show colonies on the surface of irradiated chicken skin samples treated with TSP plus lysozyme or TSP/ osmotic shock plus lysozyme, respectively. The number and size of bacterial colonies were much reduced when compared to the appropriate control (Fig

4.3.2). Results for colony counts show a synergistic interaction between all components of the TSP, osmotic shock, lysozyme treatment at 37 °C (Fig. 4.3.5) with a 90 % reduction in the count for the combined treatment. TSP alone or in combination with lysozyme, produced kills of 25 - 50 %. The smaller size of colonies in combined treatments (see Figs. 4.3.3 and 4.3.4) compared to the control (Fig 4.3.2), suggest that even where cells survive treatment, they are damaged and growth after overlaying with agar is delayed. Alternatively, inoculated bacteria may not be evenly distributed over the skin surface, so that colonies on control plates derive from a number of bacteria, whereas on treated skin they generally derive from single surviving bacteria in particular locations (e.g. within surface pores, or where protected by fatty material).

In the experiments conducted using the *in situ* detection method, the chicken skin was irradiated to kill endogenous bacteria. However, the method might be successfully used with non-irradiated foodstuffs to detect total bacteria (capable of aerobic growth) or of specific bacterial groups using selective agar overlays, for example, MacConkey agar to isolate specifically enteric bacteria.

Fig. 4.3.1. The effect of TSP (0.005 M), osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at room temperature. Untreated, uninoculated chicken skin.

Post-treatment survival was detected by the *in situ* NBT method.

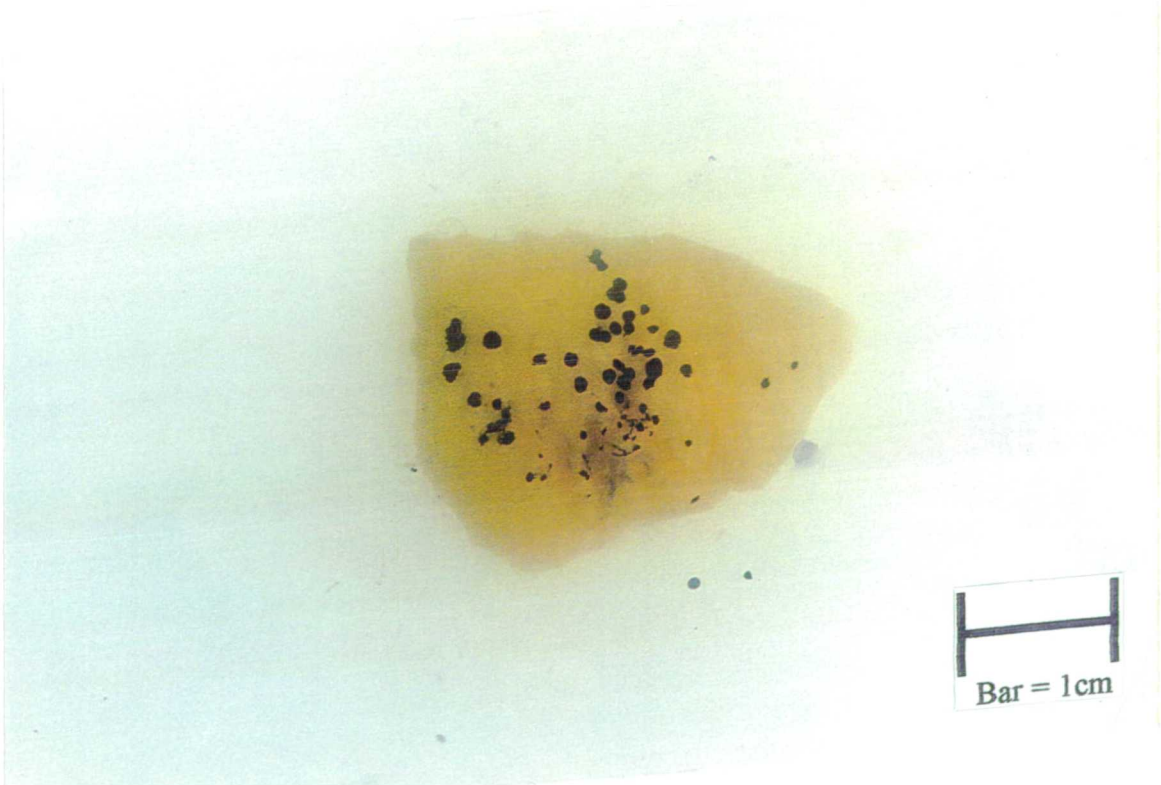


Fig. 4.3.2. The effect of TSP (0.005 M), osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at room temperature. Inoculated, untreated chicken skin.

Post-treatment survival was detected by the *in situ* NBT method.

Fig. 4.3.3. The effect of TSP (0.005 M), osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at room temperature. Inoculated, chicken skin treated with TSP and lysozyme.

Post-treatment survival was detected by the *in situ* NBT method.

Fig. 4.3.4. The effect of TSP (0.005 M), osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at room temperature. Inoculated, chicken skin, treated with TSP/NaCl/lysozyme.

Post-treatment survival was detected by the *in situ* NBT method.

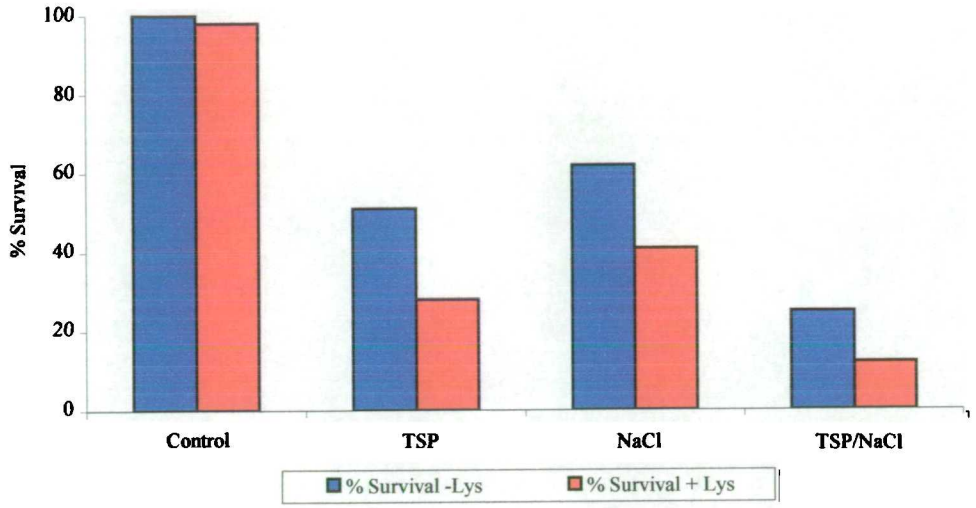


Fig. 4.3.5. The effect of a low concentration of TSP (0.005 M), osmotic shock and lysozyme on the survival of *E. coli* attached to chicken skin at room temperature. Post-treatment survival was detected by the *in situ* NBT method.

Irradiation chicken skin samples (1 g) inoculated with 20 µl of *E. coli* culture were dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP (0.005 M), TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature (22 °C), the samples were transferred to deionised water or deionised water containing 100 µg ml^{-1} lysozyme (10 ml). After incubation for a further 10 min on a rotary shaker (100 rpm), samples were transferred to petri dishes and survivors detected on the chicken skin surface by overlaying with agar and visualising with NBT after overnight incubation.

4.3.2.8. The combined effect of nisin and lysozyme in TSP and osmotic shock/TSP killing procedures for bacteria attached to chicken skin.

Nisin is a bacteriocin which acts against the cell membrane and is most active against Gram +ve bacteria. However, Gram -ve bacteria become susceptible if the outer membrane of the wall is damaged (Carniero de Melo, 1997). It appeared possible that TSP/osmotic shock procedures might allow the passage of nisin to the cell membrane. Therefore, experiments were conducted to investigate the potential for combining lysozyme and nisin in the rinsing step of the TSP/osmotic shock combined treatment, developed in this thesis (Section 4.3).

The experimental design was based on that used previously in Section 4.3. Briefly, chicken skin was inoculated with *E. coli* culture and shaken for 10 min at room temperature in either TSP solution or TSP containing 0.8 M NaCl. The skin was then removed from TSP solution and shaken for a further 30 min at room temperature (22 °C) or 37 °C, in either deionised water or deionised water containing lysozyme and nisin. The concentrations of lysozyme and nisin used in these experiments were 100 μg ml^{-1} and 34 μM respectively. The concentration of lysozyme was that used previously in this thesis. The concentration of nisin was as recommended by Carneiro de Melo (1997), who investigated the use of TSP/nisin treatments to kill Gram -ve bacteria.

In previous experiments conducted in this section of the thesis, TSP combination treatments incorporating a lysozyme rinsing step, reduced bacterial populations on chicken skin by ≥ 95 % at TSP concentrations of between 0.002 M and 0.01 M. The

effect of lysozyme plus nisin in the rinsing step, at both ambient temperature and 37 °C (Table 4.3.11), produced kills of up to 95 %. These experiments suggested that nisin would not enhance lysozyme-induced kills of Gram -ve bacteria and this line of investigation was not pursued further. It is possible that nisin and lysozyme may act antagonistically under these conditions or that they affect the same population of cells, i.e. if the outer membrane of the cell wall is damaged, the presence of nisin or lysozyme will result in cell death. The use of nisin may, however, extend the range of target bacteria to include Gram +ve bacteria (see Section 1.8.2.2.2).

Table 4.3.11. The combined effect of nisin and lysozyme on the survival of *E. coli* attached to chicken skin treated with TSP or TSP plus NaCl. Experiments were conducted at ambient temperature (Table A) or at 37 °C (Table B).

Chicken skin samples (1 g) were inoculated with an early stationary phase *E. coli* culture and dried for 30 min in air at ambient temperature (22 °C). Inoculated test samples were transferred to TSP, TSP plus 0.8 M NaCl, 0.8 M NaCl or sterile deionised water (10 ml). After incubation for 10 min on a rotary shaker (100 rpm) at ambient temperature (22 °C) or 37 °C, the samples were transferred to deionised water or deionised water containing lysozyme (100 μg ml^{-1}) plus nisin (34 μM) (10 ml). After further incubation for 10 min on a rotary shaker (100 rpm), samples were stomached individually for 2 min. Serial dilutions of stomached samples were plated on nutrient agar.

A. Ambient temperature (22 °C).

NaCl (M)	TSP (M)	pH*	Nisin (34 μM) + Lysozyme (100 μg/ml)	% Cell Survival
none	none	6.68	-	100
0.8	none	6.24	+	81
none	0.005	9.93	-	27
0.8	0.005	9.87	+	23
none	0.01	10.82	-	21
0.8	0.01	10.76	+	5.0
none	0.02	11.19	-	47
0.8	0.02	11.32	+	21
none	0.05	11.66	-	71
0.8	0.05	11.67	+	16
none	0.10	11.88	-	57
0.8	0.10	11.88	+	21

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water, at the end of experiments.

Continued

Table 4.3.11. Continued.

(B) 37 °C.

NaCl (M)	TSP (M)	pH*	Nisin (34 μM) + Lysozyme (100 μg/ml)	% Cell Survival
none	none	6.66	-	100
0.8	none	6.80	+	15
none	0.005	10.80	-	21
0.8	0.005	10.54	+	48
none	0.01	11.16	-	100
0.8	0.01	11.20	+	16
none	0.02	11.47	-	47
0.8	0.02	11.46	+	27

*pH of initial treatment solutions, i.e. TSP, NaCl, TSP + NaCl, or water, at the end of experiments.

4.3.3. SUMMARY AND CONCLUSIONS

The novel food decontamination treatment studies reported in Section IV have attempted to determine the potential of trisodium phosphate (TSP) combined with osmotic shock/cold shock/lysozyme treatments for the killing of food-borne human pathogens and spoilage organisms. TSP was first proposed as a food decontaminant in the patent of Bender and Brotsky (1992). This patent described a process whereby poultry carcasses were treated with a TSP solution of between 4 % and 12 % w/v (pH ≥ 12.5). Rhône Poulenc subsequently marketed the TSP poultry treatment under the name of AvGard. The potential use of TSP for the bacterial decontamination of poultry and meat has since been the subject of a number of studies (Bender & Brotsky, 1992; Coppen, 1993; Kim & Slavik, 1994a; Kim *et al.*, 1994; Lillard, 1994; Slavik *et al.*, 1994; Dickson *et al.*, 1994; Slavik *et al.*, 1994; Hwang & Beuchat, 1995). TSP (10 % w/w) reduced the numbers of *Salmonella* by 1.6 to 1.8 log cycles on chicken carcasses (Slavik *et al.*, 1994; Kim *et al.*, 1994), and the numbers of *E. coli* O157:H7 and *S. typhimurium* by 1.4 and 0.9 log cycles respectively, on fat surfaces of beef tissue (Kim and Slavik, 1994b). Recently Morris *et al.* (1997) used TSP to reduce *S. typhimurium* on artificially inoculated pork skin; they reported that treating pork skin with ≥ 8 % TSP reduced the *S. typhimurium* population by 2 log cycles.

The TSP concentration recommended in the AvGard decontamination procedure (Anon., 1992) is high (0.4 M; pH 12.0); such a high concentration may limit the acceptability of the TSP treatment for poultry and other foods. Additionally, the recommended TSP procedure is reported to be ineffective against Gram +ve spoilage bacteria and appears

to have limited effectiveness against certain Gram -ve bacteria, for example *Campylobacter* (Slavik *et al.*, 1994). Interestingly, control experiments conducted in Section 4.2 showed that approximately 1 % of *E. coli* attached to chicken skin survived very high TSP concentrations for > 30 min and, following washing, were recoverable in wash fluids. The mechanism of TSP action is believed to be partially attributable to its surfactant properties. This suggested the possibility that TSP treatments might act synergistically with osmotic shock treatments in damaging the outer membrane of Gram -ve bacteria and allowing entry of lysozyme.

Initial experimental studies (Section 4.1) determined the optimal *in vitro* conditions for killing cell suspensions of representative Gram -ve (*E. coli, C. jejuni, S. enteritidis and P. fluorescens*) and Gram +ve organisms (*L. monocytogenes, Staph. aureus*). These experiments determined that high kills (>99 %) of suspended Gram -ve organisms could be obtained in combined TSP/osmotic shock/lysozyme treatments and that the TSP concentrations required were low (≤ 5 mM). Significantly, the combined killing procedures developed did not appear markedly dependent upon temperature (4 - 37 °C). These studies also showed that *L. monocytogenes* was more susceptible to the combined treatment than to TSP alone (Bender & Brotsky, 1992).

In any application of the combined TSP/osmotic shock/lysozyme treatment to the killing of bacteria on foods, it is likely that treatment fluids and/or the food surface would become contaminated with organic matter. Therefore, cell killing experiments were conducted in the presence of 50 % v/v serum. These experiments showed that cells were protected by the organic material. However, this protective effect could be

overcome by increasing the TSP concentration from approximately 0.001 M to 0.01 M (0.4 % w/v). The TSP concentration required was still low compared to the concentrations (8 - 12 % w/v) used in TSP-only treatments (Bender & Brotsky, 1992; Coppen, 1993; Kim & Slavik, 1994a; Hwang & Beuchat, 1995).

The final part of the study (Section 4.3) involved the application of combined TSP-killing procedures, originally optimised using cells in suspension, against pathogenic or food-spoilage organisms artificially attached to foods and selected food-contact surfaces. After treatment, the extent of bacterial survival on foods was determined using a standardised stomaching method (British Poultry Meat Association Ltd.; Parry *et al.*, 1982). The *in situ* NBT detection method was also used to detect bacterial survival on food contact surfaces and, in a small number of experiments, irradiated chicken skin samples.

Initial experiments with TSP combination treatments and artificially inoculated chicken skin were conducted with TSP concentrations of 0.01 - 0.05 M (see Section 4.3). However, only modest bacterial kills (≤ 50 %) were obtained, much lower than those for suspended cells (≥ 99 %). Similarly, low kills were obtained for *E. coli* attached to prawns. However, high kills were obtained for *S. enteritidis* attached to lettuce leaf or food contact surfaces (ceramic, stainless steel).

Thus, it appeared that the low kills on the chicken skin and prawn surfaces were not attributable to bacterial attachment *per se* but were due to the nature of the surface. It appeared possible that cells might become lodged within crevices or pores on the skin or prawn surface, or be protected by fat, proteins or other organic material. However, it was also possible that reduced kills on chicken skin and prawn surfaces were due to the difficulty of effectively rinsing these surfaces after TSP treatment and the carryover of TSP to the lysozyme solution. Lysozyme has lower activity at high pH (pH 9; Yang and Cunningham, 1993). Carryover appeared potentially substantial and it was estimated that it could amount to as much as 0.3 g of treatment fluid/rinse water per g of chicken skin. In addition, if the TSP solutions formed a layer at the skin surface, the pH within the layer might be substantially higher than that in the bulk lysozyme solution. Therefore, the efficacy of lower TSP concentrations (1 and 10 mM) in combined treatments was investigated (Section 4.3). In these experiments, kills of skin-attached *E. coli* were ≥ 95 %. High kills were also evident using the *in situ* NBT method to determine the numbers of survivors (Section 4.2).

The maximal cell kills of *E. coli* obtained on chicken skin using TSP (1-10 mM) combined treatments were comparable to those previously published for chicken skin contaminated with *S. typhimurium* and treated with high TSP concentrations (10 % w/v; 0.4 M) of TSP (Slavik *et al.*, 1994; Kim *et al.*, 1994; Tamblyn *et al.*, 1997).

SECTION V.

General Discussion and Future Work.

The microbial contamination of food and food-contact surfaces is an ever-increasing concern within the food industry worldwide and is recognised as the most important factor compromising food quality and safety.

5.1. Bacterial survival on food and food-contact surfaces.

The *in situ* NBT method for the detection of adhered organisms on surfaces was evaluated for its suitability and applicability in Section III of this thesis. The method involved a simple procedure whereby samples of test surfaces were overlaid with agar and, after incubation, colonies were visualised by reaction with nitroblue tetrazolium to produce bright purple colonies. The method enabled evaluation of microbial survival on a diverse range of surfaces including stainless steel, white ceramic, wood and chicken skin. In all cases, following reaction with NBT, colonies could be readily visualised.

The *in situ* method was considered an improvement over conventional recovery techniques (Niskanen and Pohja, 1977), because enumeration of viable organisms took place on the original contaminated surface. This avoided the necessity for removing the surviving bacteria by agar contact or by swabbing, and subsequently plating them on agar media. Thus, when compared to a conventional swabbing technique, recoveries were >5 fold higher using the *in situ* method. The *in situ* method would also be predicted to be more reproducible than conventional methods because it detects surviving bacteria on surfaces, regardless of how firmly they are attached. In contrast, the numbers of bacteria recovered by conventional techniques will be reduced by any failure to detach organisms from the surface (Niskanen and Pohja, 1977). In addition, in swabbing techniques using swabs moistened in a resuscitation medium, organisms which have been detached into the medium may not be completely recovered from the test surface. The number of bacteria recovered may also be further reduced by inefficient removal of organisms bound to swabs and, possibly, death of organisms as a result of

additional hydration-dehydration stresses imposed; in this study, drying of bacteria was frequently associated with large reductions in viability.

Interestingly, some recent studies have adopted a variation on the NBT detection method reported here. Frank and Chmielewski (1997) assessed the effectiveness of cleaning surfaces (stainless steel, polycarbonate and mineral resin) with quaternary ammonium compounds or chlorine by allowing detergent-treated surfaces to dry for 1 h; following drying, the surfaces were coated with sterile plate count agar supplemented with potassium tellurite. The coated surfaces were incubated in sterile petri dishes for 48 h and black colonies on the test surface were enumerated. Welker *et al.* (1997) investigated the retention and cleanability of bacteria attached to plastic and wooden cutting boards. Bacterial recovery was carried out by overlaying the dry, inoculated surfaces with Luria-Bertani agar containing X-gal. The surfaces were incubated for 48 h and then the number of blue-coloured (ß - galactosidase producing) colonies were counted directly. The use of such *in situ* detection techniques may provide a reliable approach to the standardisation of future research concerning survival on surfaces.

The *in situ* NBT method was used in this study with a range of mesophilic bacteria; however it would also appear suitable for the psychrotrophic organisms important in food spoilage, provided they possess some oxidative ability and are able to reduce NBT. Possibly, psychrotrophic organisms might be more sensitive to the temperature of molten nutrient agar (45 °C), but this may be overcome by substituting gelatin agar which is poured at a lower temperature (35 °C) (see Section 3.3).

The *in situ* method was used to systematically investigate factors affecting the survival of foodborne pathogens and spoilage organisms dried on food-contact surfaces. In the experiments conducted, the survival of a selection of Gram -ve and Gram +ve organisms (*L. monocytogenes, Staph. aureus, E. coli, S. enteritidis* and *P. fluorescens*) suspended in various fluids on a number of different food-contact surfaces was determined. In previous studies, there have been conflicting results regarding the extent of bacterial

survival on surfaces (see Section 3.2), particularly in relation to the influence of drying on bacterial survival in surface films (Helke and Wong, 1994; McEldowney and Fletcher 1988; Lemcke, 1959 and Humphrey *et al.*, 1995). The present study showed that bacteria survived well in liquid films but high reductions in viability occurred at the point of drying. In dried films, there was a relatively slow further decline in viability.

The influence of temperature and relative humidity on bacterial survival on surfaces was also investigated, as the literature is again contradictory regarding the influence of these factors (Sleesman and Leben, 1976; McEldowney and Fletcher, 1988; Palumbo and Williams, 1990; Strange and Cox, 1976; Jawad *et al.*, 1996 and Helke and Wong, 1994). In comparison to the nature of the suspending media in which cells were applied to surfaces, the present study showed that the effects of physical factors were small; however, both temperature (10 to 37 °C) and relative humidity (30 to 80 % saturation) did influence bacterial survival. In general, survival was greater at lower temperatures and relative humidity values (Sleesman and Leben, 1976; Palumbo and Williams, 1990), however, McEldowney and Fletcher (1988) showed that bacterial survival was similar at different relative humidity values ranging from 0 to 75 %. Gram -ve organisms appeared more sensitive than Gram +ve organisms to a variety of environmental stresses (desiccation, temperature, relative humidity, suspending menstrua; Skaliy and Eagon, 1972; Palumbo and Williams, 1990; Jawad *et al.*, 1996).

Several previous studies have investigated the influence of contrasting surface types on survival of attached bacteria (Helke and Wong, 1994; Ak *et al.*, 1994a, 1994b; Eginton *et al.*, 1995; Mafu *et al.*, 1990; Suárez *et al.*, 1992; Holah and Thorpe, 1990). These have produced conflicting results. In the experiments conducted in this thesis, the nature of the surfaces used (stainless steel, plastic and glass) had relatively little effect on survival, i.e. the results were in accord with those of Holah and Thorpe (1990), Mafu *et al.* (1990) and Ak *et al.* (1994a, 1994b). Also, survival rates on polished and natural mill finish stainless steel and smooth and abraded ceramic tiles were similar. Thus, minor surface

abrasion (both artificial abrasion and natural wear) would not be expected to lead to increased survival rates.

The *in situ* NBT method has also been used in an independent study to evaluate the survival of both Gram -ve and Gram +ve organisms on wood surfaces (Barnes, 1998). Previously, the investigation of bacterial survival on wooden surfaces has been fraught with difficulty and much of the research is contradictory (Ak *et al.*, 1994a; 1994b; Abrishami *et al.*, 1994; Miller *et al.*, 1996; Galluzzo and Cliver, 1996). The main difficulties appear to be the possible adsorption of water and cell suspension by the wood, consequent effects on the drying rates of bacterial suspensions, and sampling methods. Bacteriological sampling in previous studies has been based on swabbing or agar contact methods which may not recover tightly adhered organisms located beneath the surface or adsorbed into the wood. In contrast to these studies, the NBT method enabled bacterial colony growth to be observed on the surface of the wood. Previously, adhered cells have only been observed by SEM (Abrishami *et al.*, 1994; Galluzzo and Cliver, 1996), but these studies were more qualitative than quantitative. Using the *in situ* NBT method, Barnes (1998) observed that bacterial survival on beech (a representative softwood, commonly used to manufacture domestic chopping boards) was higher than on the impervious surfaces used in this thesis, perhaps reflecting increased drying rates because of water adsorption by the wood. However, on iroko (a hardwood, commonly used to manufacture domestic and commercial chopping boards) cells of Gram +ve bacteria were killed although survival rates of Gram -ve bacteria were similar to those on beech. This suggested the presence of a bactericidal substance(s) in iroko and an ethanolic extract from the wood was shown to be toxic to Gram +ve cells.

On all the test surfaces used in this study and under a variety of experimental conditions, Gram +ve bacteria (*L. monocytogenes, Staph. aureus*) generally survived better than Gram -ve bacteria (*E. coli, S. enteritidis* and *P. fluorescens*). Previous results have generally shown that Gram -ve organisms are more sensitive than Gram +ve organisms to environmental stresses (Skaliy and Eagon, 1972; Palumbo and Williams,

1990; Jawad *et al.*, 1996). The suspending media in which cells were applied to surfaces was also a key factor influencing survival. Recoveries were high for bacteria suspended in serum or sucrose; in contrast, survival in deionised water was low, particularly for Gram -ve organisms. The results with serum were considered to be of particular importance because, serum-based exudates are frequently left on food preparation surfaces after handling meat and poultry carcasses. Additional experiments have yet to be conducted to assess survival in blood, but results similar to those for serum would be expected. The NBT method might also be useful to investigate the influence fats and other food materials upon bacterial attachment and survival on food contact surfaces. Previous experiments have been conducted whereby, prior to bacterial inoculation, chicken fat was rubbed onto wooden chopping boards, to simulate the accumulation of food residues (Ak *et al.*, 1994b). In these experiments, bacterial survival was reported to be high and chicken fat was therefore considered to protect bacteria from the effects of drying in air.

In the work conducted in Section III of the thesis, the *in situ* method was also successfully used in a limited study to assess the ease with which food-contact surfaces could be decontaminated. It was determined that more than 99 % of adherent organisms present on the food-contact surfaces tested could be removed by a two minute wash with deionised water, even where the organisms had originally been applied to the surface in 10 % (v/v) serum. Industrial and household detergents both improved bacterial removal; the industrial detergent was more effective than the domestic detergent but the industrial sanitiser removed and killed all inoculated organisms. These observations would therefore support the guidance (Zottola and Sasahara, 1994) that the cleaning of surfaces coming into contact with high risk foods should include disinfection procedures. However there is a need for further studies to consider the relative ease of decontamination of abraded surfaces and surfaces contaminated with foodstuffs such as animal fat.

In conclusion, as regards bacterial survival on food contact surfaces, previous studies have reported mainly conflicting findings. This may primarily have been due to the lack of a satisfactory and reproducible testing procedure. The *in situ* NBT method used here avoids the major problems of conventional methods which are the variable and inconsistent efficiency of sampling and, in swabbing methods, increased stress associated with partial hydration and dehydration of cells. The key findings of this study are that surface type, surface abrasion, relative humidity and temperature (see Section 3.2) have little effect on survival. However, survival is greatly influenced by the nature of the suspending medium in which cells are applied to the surface, and is increased by the presence of both organic and inorganic substances. The survival of Gram +ve organisms was also much greater than that of Gram -ve organisms, especially in deionised water or dilute solutions.

5.2. Bacterial decontamination of food and food - contact surfaces.

The TSP/ osmotic shock/ lysozyme decontamination procedures developed in this thesis would appear applicable to the treatment of chicken carcasses, other food stuffs and possibly food-contact surfaces. The treatment requires two distinct steps. The first is the exposure to TSP (0.002 to 0.01 M) plus 0.8 M NaCl. The second is exposure to lysozyme (100 $\mu g\ ml^{-1}$). However, the lysozyme solution must be applied immediately after the TSP/NaCl solution. In relation to meat and poultry processing, a key advantage of this treatment would be that it does not alter organoleptic qualities or cause discoloration, unlike other proposed bactericidal treatments such as those involving organic acids (Izat *et al.* 1989) or hypochlorites (Slavik *et al.,* 1991). In addition, compared to the commercially marketed TSP treatment (AvGard, Anon, 1992), which uses up to 12 % w/v TSP, the combined treatment developed here is less likely to leave behind undesirable residues on the food surface or cause corrosion of industrial processing equipment. The maximum pH of treatment fluids is ≤ 10, in contrast to pH values >12 for the TSP-only treatment patented by Bender and Brotsky (1992). A further advantage of the TSP combined treatment methods, is that they may also reduce the population of certain pathogenic Gram +ve organisms, e.g. *L. monocytogenes*, which are relatively resistant to the TSP-only treatment. A patent for the TSP combined treatments developed here has been filed by the UK Ministry of Agriculture, Fisheries and Food (Miles *et al.*, 1997).

A major difficulty envisaged in the application of TSP combined treatments to meat and poultry processing is the accurate control and maintenance of the TSP concentration (and pH value) and the effect of carryover of TSP into lysozyme washing solutions. To obtain high bacterial kills in laboratory experiments using chicken skin, there was a relatively narrow window for the TSP concentration. During processing procedures involving dipping of carcasses in treatment fluids, the effective concentration and pH of TSP solutions would inevitably become reduced due to accumulation of organic matter from successive carcass washes. This potential problem could, however be circumvented

by spraying treatment solutions onto carcasses or meat products, rather than immersing them in treatment tanks. An independent study (Flores *et al.*, 1996) has shown that the efficacy of TSP-only treatments is improved when the phosphate is applied by spraying.

The points within the poultry processing cycle where TSP combined treatments might be integrated, with least disruption or alteration to current processing plant technology, are shown in Fig. 5.1. Following the scalding and plucking stages, carcasses are then subjected to evisceration procedures (10 min), followed by an 'inside and outside' spray wash. At this point, it would appear feasible to substitute the water wash with a TSP + NaCl spray wash followed by a lysozyme wash, so completing the TSP-combined treatment prior to chilling (at 0 - 4 °C). Fratamico *et al.* (1996) suggested that bactericidal treatment rinses should be applied to carcasses prior to chilling, because organisms injured by the treatment rinses may be further stressed by refrigerated temperatures.

TSP combined treatments might also be applicable to the decontamination of non-meat foods, for example, eggs and the salad and vegetable components of perishable processed foods. Previously, Sims *et al.* (1989) showed that cottage cheese containing peppers and onion treated with sorbic acid was less likely to support the survival and growth of *S. typhimurium, Staph. aureus, B.cereus and Y. enterocolitica.* Zhuang *et al.* (1995) and Zhuang and Beuchat (1996), also demonstrated the efficacy of chlorine and TSP treatments respectively, in the reduction of *Salmonella montevideo* on raw tomatoes. Salmonellaeand other pathogens are frequently present on the surface of commercially processed eggs (Catalano and Knabel, 1994a) which might also be a target for the application of TSP/osmotic shock/lysozyme treatments. The studies in Section 4.3 of this thesis show that , in addition to killing surface attached bacteria, TSP combined treatments are also highly effective against organisms washed off surfaces in treatment fluids. This would greatly reduce bacterial crosscontamination of eggs via recycled washwater (Catalano and Knabel, 1994b).

In relation to the use of TSP-combined treatments in cleaning of food contact surfaces, optimal exposure times, TSP concentration and temperature have yet to be defined. The treatments may appear more laborious and less effective than certain currently available treatments (e.g. with hypochlorite). Nonetheless, there appears to be an increasing interest in the potential applications of TSP as a food-contact surface decontaminant (Somers *et al.*, 1994; Korber, *et al.*, 1997). TSP, and hence the TSP combined treatments developed in this thesis, have an important advantage over current surface sanitisers used both in the home and the food processing industry. This is because they do not leave chemical traces on food preparation surfaces which may taint food and, additionally, the components of the treatments are accepted as non-toxic by the USDA (1982). They would therefore not constitute a potential health risk to the consumer. The advantages of the TSP-combined treatments, over TSP-only treatments, in relation to reduced corrosion and greater effectiveness have already been discussed.

The effectiveness of TSP/osmotic shock/lysozyme combined treatments against the endogenous bacterial populations of foodstuffs has yet to be evaluated. Previous studies (Lillard, 1994; Kim *et al.*, 1994; Morris *et al.*, 1997) have shown that high concentrations of TSP (i.e. approximately 10 % w/v) reduced the total aerobic plate counts (APC) on chicken carcasses by only approximately 1.5 $\log_{10}$ and did not reduce the APC on pork skin. The limited success of TSP-only treatments against the endogenous populations of pork and chicken skin might be attributable to the stronger surface attachment of this population compared to the transient population which includes Gram -ve pathogens, such as *Salmonella* and *Campylobacter*. Thus, the TSP Avgard process has been described as a poultry processing aid (Anon, 1992) and not as a general bacterial decontamination treatment. There is no suggestion that it has significant effects on the endogenous population and is, therefore, able to prolong shelf life. The TSP-combined treatments were similarly ineffective against *Staph. aureus*. However, *L. monocytogenes* was susceptible (Section 4.2). Thus, the treatment may be more effective against Gram +ve spoilage bacteria than TSP-only treatments. It was also shown that the TSP-combined treatment was highly effective against the Gram -ve

food spoilage organism *P. fluorescens*. Thus, any commercial trial of the TSP-combined treatments should include an assessment of their role in reducing APCs and prolonging shelflife.

In general, the future work required to promote the use of the TSP-combined killing treatments developed in this thesis are industrial level trials and/or laboratory experiments to evaluate their use on a more diverse range of foodstuffs. The effectiveness of the procedures also needs to be assessed against a wider range of target organisms, including populations naturally present on food. Modifications to the procedures developed might also further enhance killing efficacy. Hen egg lysozyme (14,600 Da) has the lowest molecular weight of those commercially available (Jolles *et al.*, 1963). Thus, it would be expected to be the most readily transported across the damaged outer membrane of Gram -ve bacteria. However, Ibrahim *et al.* (1994) have developed a recombinant lysozyme molecule with a hydrophobic pentapeptide at its C terminus. The enzyme was expressed in a yeast system and has enhanced bactericidal properties against *E.coli*. This was partially attributed to the conformational changes of the recombinant enzyme (partially unfolded) which may have facilitated enzyme penetration of the outer membrane of the cell wall. This modified lysozyme might therefore be more suitable than hen egg lysozyme in TSP/osmotic shock killing procedures.

It is also possible that bacteriocins may be more effective than lysozyme against membrane-damaged cells. Carniero de Melo (1997) used the TSP/osmotic shock treatments developed here to disrupt the outer membrane of Gram -ve cells, prior to nisin treatment. Kills with nisin were as good, or better, than with lysozyme. In addition, nisin was also effective against Gram +ve bacteria and rapidly killed *Staph. aureus* (resistant to lysozyme combined treatments). However, although nisin is commercially available, it is relatively expensive compared to lysozyme, for which a crude egg white extract may be substituted (Chatzopoulou, 1991). Nevertheless, relative costs may change with the increasing use of nisin in the food industry. Also, the

range of known bacteriocins is continually being increased. This has revealed bacteriocins with contrasting antibacterial spectra. It therefore appears probable that in the future, TSP/osmotic shock treatments combined with one or more bacteriocin may enable development of decontamination techniques effective against virtually all bacteria.

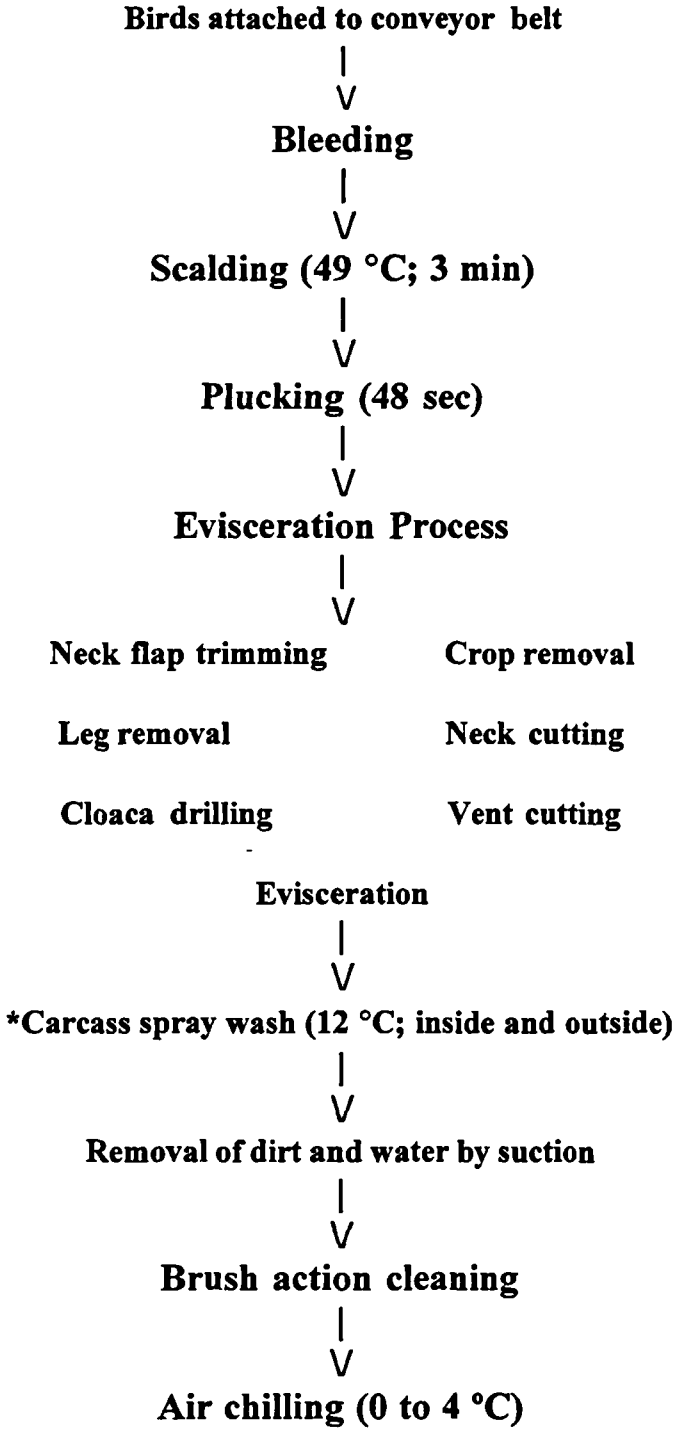


Fig. 5.1. Layout and operating conditions of a typical poultry processing plant. (Chatzopoulou, 1991).

* Proposed stage for incorporation of TSP+NaCl and lysozyme treatments.

SECTION VI.

Appendix

Appendix 6.1.

Table 6.1. The effect of a domestic dish detergent (Fairy Liquid) on the survival of *Staph. aureus* and *S. enteritidis*.

Broth cultures were diluted by a factor 1:100 into detergent solutions at 37 °C and incubated for 2 min in a shaking incubator. The initial cell concentrations in detergent solutions were approximately 3.0×10^7 cfu ml^{-1}. There was no killing effect when cells were diluted into deionised water.

Organism	% survival concentration of domestic detergent (% v/v)					
	0.0	0.1	0.2	0.5	1.0	2.0
Staph. aureus	100	100	100	90	64	11.5
S. enteritidis	100	100	100	100	100	83

Table 6.2. The effect of an industrial detergent (Liquid Multiclean SU 125) on the survival of *Staph. aureus* and *S. enteritidis*.

Broth cultures were diluted by a factor 1:100 into detergent solutions at 37 °C and incubated for 2 min in a shaking incubator. The initial cell concentrations in detergent solutions were approximately 3.0×10^7 cfu ml^{-1}. There was no killing effect when cells were diluted into deionised water.

Organism	% survival concentration of an industrial detergent (% v/v)					
	0.0	0.1	0.2	0.5	1.0	2.0
Staph. aureus	100	23	12	2.5	0	
S. enteritidis	100	100	83	16	23	21

Table 6.3. The effect of an industrial sanitiser (Quatdet SU 321) on the survival of *Staph. aureus* and *S. enteritidis*.

Broth cultures were diluted by a factor 1:100 into detergent solutions at 37 °C and incubated for 2 min in a shaking incubator. The initial cell concentrations in detergent solutions were approximately 3.0×10^7 cfu ml^{-1}. There was no killing effect when cells were diluted into deionised water.

Organism	% survival concentration of industrial sanitiser (% v/v)				
	0.0	0.1	0.2	0.5	1.0
Staph. aureus	100	0	0	0	0
S. enteritidis	100	10	1.0	1.0	0

SECTION VII.

References

Abrishami, S.H., Tall, B.D., Bruursema, T.J., Epstein, P.S. and Shah, D.B. (1994) Bacterial adherence and viability on cutting board surfaces. *Journal of Food Safety* **14,** 153-172.

Abu-Amero, K.K., Halabalab, M.A. and Miles, R.J. (1996). Nisin resistance distinguishes *Mycoplasma* spp. from *Acholeplasma* spp. and provides a basis for selective growth media. *Applied and Environmental Microbiology* **62,** 3107-3111.

Ahern, T.J. and Klibanov, A.M. (1985). The mechanism of irreversible enzyme inactivation at 100 °C. *Science* **228,** 1280-1284.

Ak, N.O., Cliver, D.O. and Kaspar, C.W. (1994a) Cutting boards of plastic and wood contaminated experimentally with bacteria. *Journal of Food Protection* **57,** 16-22.

Ak, N.O., Cliver, D.O. and Kaspar, C.W. (1994b) Decontamination of plastic and wooden cutting boards for kitchen use. *Journal of Food Protection* **57,** 23-30.

Al-Makhlafi, H., Mcguire, J. and Daeschel, M. (1994) Influence of preadsorbed milk proteins on adhesion of *Listeria monocytogenes* to hydrophobic and hydrophilic silica surfaces. *Applied and Environmental Microbiology* **60,** 3560-3565.

Al-Makhlafi, H., Nasir, A., Mcguire, J. and Daeschel, M. (1995) Adhesion of *Listeria monocytogenes* to silica surfaces after sequential and competitive adsorption of bovine serum albumin and β-lactoglobulin. *Applied and Environmental Microbiology* **61,** 2013-2015.

AlteKruse, S.F. and Swerdlow, D.L. (1996) The changing epidemiology of foodborne diseases. *The American Journal of the Medical Sciences* **311,** 23-29.

Anderson, M.E. and Marshall, R.T. (1989) Interaction of concentration and temperature of acetic acid solution on reduction of various species of microorganisms on beef surfaces. *Journal of Food Protection* **52,** 312-315.

Anderson, M.E., Marshall, R.T., Naumann, H.D. and Stringer, W.C. (1975) Physical factors that affect removal of yeasts from meat surfaces with water sprays. *Journal of Food Science* **40,** 1232-1235.

Angelotti, R. Wilson, J.L., Litsky, W. and Walter, W.G. (1964) Comparative evaluation of the cotton swab and rodac methods for the recovery of *Bacillus subtilis* spore contamination from stainless steel surfaces. *Health Laboratory Science* **1,** 289-296.

Anon. (1988) Method of test for the antimicrobial activity of disinfectants in food hygiene. DD177. London: British Standards Institution.

Anon. (1992) AvGard™ TSP *Salmonella* reduction process. Rhône-Poulenc, Princeton, NJ, USA.

Anon. (1993a) Code of Federal regulations. Title 9. Animals and animal products. Ch. III, Part 318.7. Office of the Federal Register, National Archives and Records Administration, Washington, DC.

Anon. (1993b) Code of Federal regulations. Title 21. Food and drugs. Ch. I, Part 184. Office of the Federal Register, National Archives and Records Administration, Washington, DC.

Ayres, J.C. (1955) Microbiological implications in handling, slaughtering and dressing of meat animals. *Advances in Food Research* **6**, 109-161.

Baird-Parker, A.C. (1994) Food and microbiological risks. *Microbiology* **140**, 687-695.

Banks, M.K. and Bryers, J.D. (1991) Bacterial species dominance within a binary culture biofilm. *Applied and Environmental Microbiology* **57**, 1974-1979.

Barnes, B. I., Cassar, C. A., Halabalab, M. A., Parkinson, N. H. and Miles, R. J. (1996) An *in situ* method for determining bacterial survival on food preparation surfaces using a redox dye. *Letters in Applied Microbiology* **23**, 325-328.

Barnes, B.I. Studies related to the bacteriological contamination of kitchen surfaces. Thesis, University of London. To be submitted.

Bean, N.H. and Griffin, P.M. (1990) Foodborne disease outbreaks in the United States, 1973-1987: pathogens, vehicles and trends. *Journal of Food Protection* **53**, 804-817.

Becker, M.E. and Hartsell, S.E. (1954) Factors affecting bacteriolysis using lysozyme in dual enzyme systems. *Archives of Biochemistry and Biophysics* **53**, 402-410.

Becker, M.E. and Hartsell, S.E. (1955) The synergistic action of lysozyme and trypsin in bacteriolysis. *Archives of Biochemistry and Biophysics* **55**, 257-269.

Bender, F.G. and Brotsky, E. (1991) Process for treating poultry carcasses to control salmonellae growth. U.S. Patent 5, 069, 922. December 3, 1991. Int. Cl.^{5} A23L 3/34; A23L 3/34; A23L 1/135.

Bender, F.G. and Brotsky, E. (1992) Process for treating poultry carcasses to control salmonellae growth. U.S. Patent 5, 143, 739. Sep. 1, 1992. Int. Cl.^{5} A23L 3/34; A22C 21/00.

Benedict, R.C., Schultz, F.J. and Jones, S.B. (1991) Attachment and removal of *Salmonella* spp. on meat and poultry tissues. *Journal of Food Safety* **11**, 135-148.

Berg, J.D., Roberts, P.V. and Matin, A. (1986) Effect of chlorine dioxide on selected membrane functions of *Escherichia coli*. *Journal of Applied Bacteriology* **60**, 213-220.

Bester, B.H. and Lombard, S.H. (1990) Influence of lysozyme on selected bacteria associated with Gouda cheese. *Journal of Food Protection* **53**, 306-311.

Bianchi, A., Ricke, S.C., Cartwright, A.L and Gardner, F.A. (1994) A peroxidase catalysed chemical dip for the reduction of *Salmonella* on chicken breast skin. *Journal of Food Protection*, **57**, 301-304.

Blackman, I. C. and Frank, J.F. (1996) Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *Journal of Food Protection* **59**, 827-831.

Bloomfield, S.F. and Scott, E. (1997) Cross-contamination and infection in the domestic environment and the role of chemical disinfectants. *Journal of Applied Microbiology* **83**, 1-9.

Bloomfield, S.F., Arthur, M., Van Klingeren, B., Pullen, W., Holah, J.T. and Elton, R. (1994) An evaluation of the repeatability and reproducibility of a surface test for the activity of disinfectants. *Journal of Applied Bacteriology* **76**, 89-94.

Borneff, J., Hassinger, R., Wittig, J. and Edenharder, R. (1988) Distribution of microorganisms in household kitchens I. Communication: Problems, Experiments, Results. *Zentalblatt für Bakteriologie, Mikrobiologie und Hygiene* **186**, 1-29.

Borneff, J., Hassinger, R., Wittig, J. and Edenharder, R. (1988) Distribution of microorganisms in household kitchens 2. Communication: Critical evaluation of the results and conclusions. *Zentalblatt für Bakteriologie, Mikrobiologie und Hygiene* **186**, 30-44.

Bourion, F. and Cerf, O. (1996) Disinfection efficacy against pure-culture and mixed population biofilms of *Listeria innocua* and *Pseudomonas aeruginosa* on stainless steel, teflon® and rubber. *Sciences des Aliments* **16**, 151-166.

Bower, C.K., McGuire, J. and Daeschel, M.A.(1995) Suppression of *Listeria monocytogenes* colonisation following adsorption of nisin onto silica surfaces. *Applied and Environmental Microbiology* **61**, 992-997.

Bower, C.K., McGuire, J. and Daeschel, M.A. (1996) The adhesion and detachment of bacteria and spores on food-contact surfaces. *Trends in Food Science and Technology* **7**, 152-157.

Breen, P.J., Compadre, C.M., Fifer, E.K., Saliri, H., Serbus, D.C. and Lattin, D.L. (1995) Quaternary ammonium compounds inhibit and reduce the attachment of viable *Salmonella typhimurium* to poultry tissues. *Journal of Food Science* **60**, 1191-1196.

Bremner, A. and Johnston, M. (1996) Poultry meat hygiene and inspection. London: WB Saunders Company Ltd.

Bright, J.J. and Fletcher, M. (1983) Amino acid assimilation and electron transport system activity in attached and free-living marine bacteria. *Applied and Environmental Microbiology* **45**, 818-825.

Brown, C.M., Ellwood, D.C. and Hunter, J.R. (1977) Growth of bacteria at surfaces: Influence of nutrient limitation. *FEMS Microbiology Letters* **1**, 163-166.

Brown, M.R.W., Allison, D.G. and Gilbert, P. (1988) Resistance of bacterial biofilms to antibiotics: a growth related effect? *Journal of Antimicrobial Chemotherapy* **22**, 777-780.

Buchanan, R.L. and Palumbo, S.A. (1985) *Aeromonas hydrophila* and *Aeromonas sorbia* as potential food poisoning species: a review. *Journal of Food Safety* **7**, 15-29.

Bundy, J.G., Wardell, J.L., Campbell, C.D., Killham, K. and Paton, G.I. (1997) Application of bioluminescence-based microbial biosensors to the ecotoxicity assessment of organotins. *Letters in Applied Microbiology* **25**, 353-358.

Burchard, R.P.(1981) Gliding motility of prokaryotes: Ultrastructure, physiology and genetics. *Annual Reviews in Microbiology* **35**, 497-529.

Busscher, H.J. and Weerkamp, A.H. (1987) 'Specific and non-specific interactions in bacterial adhesion to solid substrata' in *FEMS Microbiological Reviews* **46**, 165-173.

Butler, J.L., Stewart, J.C., Vanderzant, C., Carpenter, Z.L. and Smith, G.C. (1979) Attachment of microorganisms to pork skin and surfaces of beef and lamb carcasses. *Journal of Food Protection*, **42**, 401-406.

Cano, R.J. and Colomé, J.S. (1988) *Essentials of Microbiology.* New York: West Publishing Company.

Carniero de Melo. A.M. Mode of action of nisin and application to food preservation. Thesis, University of London. 1997.

Carter, A.O., Borczyk, A.A., Carlson, A.K., Harvey, B., Hockin, J.C., Karmali, M.A., Krishnan, C., Korn, D.A. and Lior, H. (1987) A severe outbreak of *Escherichia coli* O157: H7-associated hemorrhagic colitis in a nursing home. *New England Journal of Medicine* **317**, 1496-1500.

Catalano, C.R. and Knabel, S.J. (1994a) Incidence of *Salmonella* in Pennsylvania egg processing plants and destruction by high pH. *Journal of Food Protection* **57**, 587-591.

Catalano, C.R. and Knabel, S.J. (1994b) Destruction of *Salmonella enteritidis* by high pH and rapid chilling during simulated commercial egg processing. *Journal of Food Protection* **57**, 592-595.

Chamberlain, A.H.L. and Johal, S. (1987) Biofilms on meat processing surfaces. In: *Biodeterioration 7,* ed. D.R. Houghton, R.N. Smith and H.O.W. Eggins, pp 57-61. London and New York: Elsevier Applied Science.

Chamberlain, A.N., Halabalab, M.A., Gould, D.J. and Miles, R.J. (1997) Distribution of bacteria on hands and the effectiveness of brief and thorough decontamination

procedures using non-medicated soap. *Zentralblatt für Bakterologie, Parasitenkunde, Infektionskranheiten und Hygiene I.Abteilung Originale* **285**, 565-575.

Chapman, P.A., Wright, D.J. and Norman, P. (1989) Verotoxin-producing *Escherichia coli* infections in Sheffield: Cattle as a possible source. *Epidemiology and Infection* **102**, 439-445.

Characklis, W.G. and Cooksey, K.E. (1983) Biofilms and microbial fouling. *Advances in Applied Microbiology* **29**, 93-137.

Chaturvedi, S.K. and Maxcy, R.B. (1969) Ecosystems of food-contact surfaces. *Food Technology* **23**, 67-71.

Chatzopoulou, A. Destruction of Gram -negative bacteria by lysozyme. Thesis, University of London, 1991.

Chatzopoulou, A. and Miles, R.J. (1992) Destruction of Gram-negative bacteria by lysozyme. In '*Salmonella* and Salmonellosis', Reports and communications of the Ploufragan symposium, pp. 399-400, Guivarch, Saint Brieuc, France.

Chatzopoulou, A., Miles, R. and Anagnostopoulos, G. (1993) Destruction of Gram-negative bacteria. Int. Pat. Appl. WO 93/00822.

Cherrington, C.A., Hinton, M., Mead, G.C. and Chopra, I. (1991) Organic acids: chemistry, antibacterial activity and practical applications. In *Advances in Microbial Physiology* Vol. 32, ed. Rose, A.H. and Tempest, D.W. London: Academic Press.

Chung, K.T., Dickson, J.S. and Crouse, J.D. (1989) Attachment and proliferation of bacteria to meat. *Journal of Food Protection*, **52**, 173-177

Clavero, M.R.S, Monk, J.D, Beuchat, L.R, Doyle, M.P. and Brackett, R.E. (1994) Inactivation of *Escherichia coli* 0157:H7, salmonellae and *Campylobacter jejuni* in raw ground beef by gamma-irradiation. *Applied and Environmental Microbiology* **60**, 2069-2075.

Colobert, L. (1957). Destruction par le lysozyme ápres depilidation de la paroi externe des *Salmonelles* pathogenes. *Comptes Rendus* **245**, 1674-1676.

Coppen, P. P. (1993) *Salmonella* reduction in poultry processing - application of TSP process to European industry. In Proceedings of the 11th European symposyum on the Quality of Poultry Meat, pp. 524-531. Branche Francaise, World Poultry Science Association.

Costerton, J.W., Geesey, G.G. and Cheng, K.J. (1978) How bacteria stick. *Scientific American* **238**, 86-95.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annual Reviews in Microbiology* **49**, 711-745.

Cowden, J.M., Wall, P.G., Adak, G., Evans, H., Le Baigue, S. and Ross, D. (1996) Outbreaks of foodborne infectious intestinal disease in England and Wales: 1992 and 1993. In *Communicable Disease Report Review* 8, pp. R109-117. London: Public Health Laboratory Service.

Crouse, J.D., Anderson, M.E. and Naumann, H.D. (1988) Microbial decontamination and weight of carcass beef as affected by automated washing pressure and length of time of spray. *Journal of Food Protection*, **51**, 471-474.

Csonka, L.O. (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* **53**, 121-147.

Cutter, C.N. and Siragusa, G.R. (1995a) Application of chlorine to reduce *Escherichia coli* on beef. *Journal of Food Safety* **15**, 67-75.

Cutter, C.N. and Siragusa, G.R. (1995b) Population reductions of Gram-negative pathogens following treatments with nisin and chelators under various conditions. *Journal of Food Protection*, **57**, 977-983.

Cutter, C.N. and Siragusa, G.R. (1995c) Treatments with nisin and chelators to reduce *Salmonella* and *Escherichia coli* on beef. *Journal of Food Protection*, **57**, 1028-1030.

Cutter, N.C. and Dorsa, W.J. (1995) Chlorine dioxide spray washes for reducing fecal contamination on beef. *Journal of Food Protection*, **58**, 1294-1296.

D'Aoust, J. (1991) Psychrotrophy and foodborne *Salmonella*. *International Journal of Food Microbiology* **13**, 207-216.

Daeschel, M.A. (1989) Antimicrobial substances from lactic acid bacteria for use as food preservatives. *Food Technology* **43**, 164-166.

Dainty, R.H. (1985) Bacterial growth in food, a nutrient-rich environment. In Bacteria in their natural environments, ed. Fletcher, M. and Floodgate, G.D. Society for General Microbiology. London: Academic Press.

De Boer, E. and Hahné, M. (1990) Cross-contamination with *Campylobacter jejuni* and *Salmonella* spp. from raw chicken products during food preparation. *Journal of Food Protection*, **53**, 1067-1068.

De Wit, J.C., Broekhuizen, G. and Kampelmacher, E.H. (1979) Cross contamination during the preparation of frozen chickens. *Journal of Hygiene, Cambridge* **82**, 27-32.

Delves-Broughton, J. (1990). Nisin and its uses. *Food Technology* **44**, 100-117.

Dexter, S.C., Sullivan, J.D., Jr., Williams I, J. and Watson, S.W. (1975) Influence of substrate wettability on the attachment of marine bacteria to various surfaces. *Applied Microbiology* **30**, 298-308.

Dhir, V.K. and Dodd, C.E. (1995) Susceptibility of suspended and surface-attached *Salmonella enteritidis* to biocides and elevated temperatures. *Applied and Environmental Microbiology* **61**, 1731-1738.

Dickson, J.S, Nettles Cutter, C.G. and Siragusa, G.R. (1994) Antimicrobial effects of trisodium phosphate against bacteria attached to beef tissue. *Journal of Food Protection*, **57**, 952-955.

Dickson, J.S. (1988) Reduction of bacteria attached to meat surfaces by washing with selected compounds. *Journal of Food Protection*, **51**, 869-873.

Dickson, J.S. (1991) Attachment of *Salmonella typhimurium* and *Listeria monocytogenes* to beef tissue: effects of inoculum level, growth temperature and bacterial culture age. *Food Microbiology*, **8**, 143-151.

Dickson, J.S. (1992) Acetic acid action on beef tissue surfaces contaminated with *Salmonella typhimurium*. *Journal of Food Science* **57**, 297-301.

Dickson, J.S. and Frank, J.F. (1993) Bacterial starvation stress and contamination of beef. *Food Microbiology* **10**, 215-222.

Dickson, J.S. and Koohmaraie, M. (1989) Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Applied and Environmental Microbiology* **55**, 832-836.

Dickson, J.S. and Macniel, M.D. (1991) Contamination of beef tissue surfaces by cattle manure inoculated with *Salmonella typhimurium* and *Listeria monocytogenes*. *Journal of Food Protection*, **54**, 102-104.

Dickson, J.S. and Siragusa, G.R. (1994) Survival of *Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* during storage on beef sanitised with organic acids. *Journal of Food Safety* **14**, 313-327.

Djuretic, T., Wall, P.G., Ryan, M.J., Evans, H.S., Adak, G.K. and Cowden, J.M. (1996) General outbreaks of infectious intestinal disease in England and Wales 1992 to 1994. In *Communicable Disease Report Review* 6, pp. R57-63. London: Public Health Laboratory Service.

Doyle, M.P. (1991) *Escherichia coli* O157: H7 and its significance in foods. *International Journal of Food Microbiology* **12**, 289-302.

Eastwood, M. (1996) Personal communication.

Eginton, P.J., Gibson, H., Holah, J., Handley, P.S. and Gilbert, P. (1995) Quantification of the ease of removal of bacteria from surfaces. *Journal of Industrial Microbiology* **15**, 305-310.

Elliot, R.P., Straka, R.P. and Garibaldi, J.A. (1964) Polyphosphate inhibition of growth of pseudomonads from poultry meat. *Applied Microbiology* **12**, 517-522.

Evans, D.J., Allison, D.G., Brown, M.R. and Gilbert, P. (1991) Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms toward ciprofloxacin: effect of specific growth rate. *Journal of Antimicrobial Chemotherapy* **27**, 177-184.

Farber, J.M. (1993) Current research on *Listeria monocytogenes* in foods: an overview. *Journal of Food Protection* **56**, 640-643.

Farber, J.M. and Idziak, E.S. (1982) Detection of glucose oxidation products in chilled fresh beef undergoing spoilage. *Applied and Environmental Microbiology* **44**, 521-524.

Fattom, A. and Shilo, M. (1984) Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. *Applied and Environmental Microbiology* **47**, 135-143.

Felix, C.W. (1993) Wooden cutting boards see new revival. *Food Protection Report* **9**, 8.

Firstenberg-Eden, R., Notermans, S. and Van Schothorst, M. (1978) Attachment of certain bacterial strains to chicken and beef meat. *Journal of Food Safety* **1**, 217-228.

Fletcher, M. (1977) The effect of culture concentration and age, time and temperature on bacterial attachment to polystyrene. *Canadian Journal of Microbiology* **23**, 1-6.

Fletcher, M. (1988) How do bacteria stick to solid surfaces? *Microbiological Sciences* **4**, 133-136.

Fletcher, M. (1991) The physiological activity of bacteria attached to solid surfaces. *Advances in Microbial Physiology* **32**, 53-85.

Fletcher, M. and Marshall, K.C. (1982) Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. *Applied and Environmental Microbiology* **44**, 184-192.

Fliss. I., Simard, R.E. and Ettriki, A. (1991) Comparison of three sampling techniques for microbiological analysis of meat surfaces. *Journal of Food Science* **56**, 249-251.

Flores, L.M., Sumner, S.S., Peters, D.L. and Mandigo, R. (1996) Evaluation of a phosphate to control pathogen growth in fresh and processed meat products. *Journal of Food Protection* **59**, 356-359.

Frank, J.F. and Chmielewski, R.A. (1997) Effectiveness of sanitation with quaternary ammonium compound or chlorine on stainless steel and other domestic food-preparation surfaces. *Journal of Food Protection* **60**, 43-47.

Frank, J.F. and Koffi, R.A. (1990) Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection* **53**, 550-554.

Fratamico, P.M., Schultz, F.J., Benedict, R.C., Buchanan, R.L. and Cooke, P.H. (1996) Factors influencing attachment of *Escherichia coli* 0157:H7 to beef tissues and removal using selected sanitizing rinses. *Journal of Food Protection* **59**, 453-459.

Galluzzo, L. and Cliver, D.O. (1996) Cutting boards and bacteria - Oak vs. *Salmonella*. *Dairy, Food and Environmental Sanitation* **16**, 290-293.

Geesey, G.G. (1982) Microbial exopolymers: Ecological and economic considerations. *American Society for Microbiology News* **48**, 9-14.

Giese, J. (1992) Experimental process reduces *Salmonella* on poultry. *Food Technology* **46**, 112.

Giese, J. (1993) *Salmonella* reduction process receives approval. *Food Technology* **47**, 110.

Gilbert, R.J. and Maurer, I.M. (1968) The hygiene of slicing machines, carving knives and can-openers. *Journal of Hygiene, Cambridge* **66**, 439-450.

Gilbert, R.J. and Watson, H.M. (1971) Some laboratory experiments on various meat preparation surfaces with regard to surface contamination and cleaning. *Journal of Food Technology* **6**, 163-170.

Gill, C.O. (1983) Meat spoilage and evaluation of the potential storage life of fresh meat. *Journal of Food Protection* **46**, 444-452.

Gill, C.O. (1986) The control of microbial spoilage in fresh meats. In Advances in Meat Research, Vol. 2, ed. Pearson, A.M. and Dutson. London: T.R. Macmillan Publishers Ltd.

Gill, C.O. and Newton, K.G. (1980a) Growth of bacteria on meat at room temperature. *Journal of Applied Bacteriology* **49**, 315-323.

Gill, C.O. and Newton, K.G. (1980b) Development of bacterial spoilage at adipose tissue surfaces of fresh meat. *Applied and Environmental Microbiology* **39**, 1076-1077.

Glynn, M.K., Bopp, C., Dewitt, W., Dabney, P., Mokhtar, M. and Angulo, F.J. (1998) Emergence of multi-drug resistant *Salmonella enterica* serotype *typhimurium* DT 104 infections in the United States. *New England Journal of Medicine* **338**, 1333-1338.

Golden, M.H., Buchanan, R.L. and Whiting, R.C. (1995). Effect of sodium acetate or sodium propionate with EDTA and ascorbic acid on the inactivation of *Listeria monocytogenes*. *Journal of Food Safety* **15**, 53-65.

Gould, G. (1996) Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. *Journal of Food Protection* Supplement, 82-86.

Greer, G. and Dilts, B.D. (1995) Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. *International Journal of Food Microbiology* **25**, 141-151.

Grula, E.A. and Hartsell, S.E. (1957) Lysozyme in the bacteriolysis of Gram -ve bacteria. *Canadian Journal of Microbiology* **3**, 13-21.

Hancock, R.E.W. (1984) Alterations in the outer membrane permeability. *Annual Reviews in Microbiology* **38**, 237-264.

Hauben, K.J.A., Wuytack, E.Y., Soontjens, C.F. and Michiels, C.W. (1996) High-pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. *Journal of Food Protection* **59**, 350-355.

Helke D.M., Somers, E.B. and Wong, A.C.L. (1993) Attachment of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N in the presence of milk and individual milk components. *Journal of Food Protection* **56**, 479-484.

Helke, D.M. and Wong, A.C.L. (1994) Survival and growth characteristics of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-N rubber. *Journal of Food Protection* **57**, 963-968, 974.

Herald, P.J. and Zottola, E.A (1988a) Attachment of *Listeria monocytogenes* to stainless steel surfaces at various temperatures and pH values. *Journal of Food Science* **53**, 1549-1552.

Herald, P.J. and Zottola, E.A. (1988b) The use of transmission electron microscopy to study the composition of *Pseudomonas fragi* attachment material. *Food Microstructure* **7**, 53-57.

Holah, J.T. and Thorpe, R.H. (1990) Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology* **69**, 599-608.

Holah, J.T., Betts, R.B. and Thorpe, R.H. (1988) The use of direct epifluorescent microscopy (DEM) and the direct epifluorescent filter technique (DEFT) to assess microbial populations on food contact surfaces. *Journal of Applied Bacteriology* **65**, 215-221.

Holah, J.T., Higgs, C., Robinson, S., Worthington, D. and Spenceley, H. (1990) A conductance-based surface disinfection test for food hygiene. *Letters in Applied Microbiology* **11**, 255-259.

Holzapfel, W.H., Geisen, R. and Schillinger,U. (1995) Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology* **24**, 343-362.

Hood, S.K. and Zottola, E.A. (1995) Biofilms in food processing. *Food Control* **6**, 9-18.

Houtsma, P.C., de Wit, J. and Rombouts, F.M. (1996) Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures. *Journal of Food Protection* **59**, 1300-1304.

Hughey, V.L. and Johnson, E.A. (1987) Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Applied and Environmental Microbiology* **53**, 2165-2170.

Humphrey, T., Mason, M. and Martin, K. (1995) The isolation of *Campylobacter jejuni* from contaminated surfaces and its survival in diluents. *International Journal of Food Microbiology*, **26**, 295-303.

Humphrey, T.J., Martin, K.W. and Whitehead, A. (1994) Contamination of hands and work surfaces with *Salmonella enteritidis* PT4 during the preparation of egg dishes. *Epidemiology and Infection* **113**, 403-409.

Hurst, A. (1981) Nisin. *Advances in Applied Microbiology* **27**, 85-123.

Hwang,C. and Beuchat, L.R. (1995) Efficacy of selected chemicals for killing pathogenic and spoilage microorganisms on chicken skin. *Journal of Food Protection*, **58**, 19-23.

Ibrahim, H.R., Yamada, M., Matsushita, K., Kobayashi,K. and Kato, A. (1994) Enhanced bactericidal action of lysozyme to *Escherichia coli* by inserting a hydrophobic pentapeptide into its C terminus. *The Journal of Biological Chemistry* **269**, 5059-5063.

IFST (1995) Current Hot Topics: *Listeria monocytogenes* in cheese. Institute of Food Science and Technology website information: http://www.easynet.co.uk/ifst/hottop5.htm

Izat, A.L., Colberg, M., Adams, M.H., Reiber, M.A. and Waldroup, P.M. (1989) Production and processing studies to reduce the incidence of salmonellae on commercial broilers. *Journal of Food Protection* **52**, 670-673.

James, C., Goksoy, E.O. and James, S.J. (1997) Past, present and future methods of meat decontamination. MAFF Fellowship in Food Process Engineering,University of Bristol.

Jawad, A., Heritage, J., Snelling, A.M., Gascoyne-Binzi, D.M. and Hawkey, P.M. (1996) Influence of relative humidity and suspending menstrua on survival of *Acinetobacter* spp. on dry surfaces. *Journal of Clinical Microbiology* **34**, 2881-2887.

Jay. J.M. (1996) Food preservation with chemicals. In Modern Food Microbiology, (5th Edition) pp. 273-275. New York: Chapman and Hall.

Johnson, B. and Moser, K. (1967) Amino acid destruction in beef by high energy electron beam irradiation. In Radiation Preservation of Foods, pp 171-179. Washington, DC: American Chemical Society.

Johnston, A.M. (1990) Veterinary sources of food-borne illness. *The Lancet* **336**, 856-858.

Jolles, J., Jaurequi-Adell, J., Bernier, I. and Jolles, P. (1963) La structure chimique du lysozyme de blanc d'ouef de poule: Etude detaille. *Biochemica et Biophysica Acta* **78**, 668-689.

Jones, M.V., Wood, M.A. and Herd, T.M. (1992) Comparative sensitivity of *Vibrio cholerae* 01 El Tor and *Escherichia coli* to disinfectants. *Letters in Applied Microbiology* **14**, 51-538.

Kelly, C.A., Dempster, J.F. and Mcloughlin, A.J. (1981) The effect of temperature, pressure and chlorine concentration of spray washing on numbers of bacteria on lamb carcasses. *Journal of Applied Bacteriology* **51**,415-424.

Kim, J. and Slavik, M.F. (1994a) Removal of *Salmonella typhimurium* attached to chicken skin by rinsing with trisodium phosphate solution: scanning electron microscopic examination. *Journal of Food Safety* **14**, 77-84.

Kim, J., Slavik, M.F., Pharr, M.D., Raben, D.P., Lobsinger, C.M. and Tsai, S. (1994) Reduction of *Salmonella* on post-chill chicken carcasses by trisodium phosphate treatment. *Journal of Food Safety* **14**, 9-17.

Kim, J.and Slavik, M.F. (1994b) Trisodium phosphate (TSP) treatment of beef surfaces to reduce *Escherichia coli* 0157: H7 and *Salmonella typhimurium*. *Journal of Food Science* **59**, 20-22.

Kim, K.Y., Frank, J.F. and Craven, S.E. (1996) Three-dimensional visualisation of *Salmonella* attachment to poultry skin using confocal scanning laser microscopy. *Letters in Applied Microbiology*, **22**, 280-282.

Klima, R.A. and Montville, T.J. (1995) The regulatory and industrial responses to listeriosis in the USA - A paradigm for dealing with emerging foodborne pathogens. *Trends in Food Science and Technology* **6**, 87-93.

Korber, D.R., Choi, A., Wolfaardt, G.M., Ingham, S.C. and Caldwell, D.E. (1997) Substratum topography influences susceptibility of *Salmonella enteritidis* biofilms to trisodium phosphate. *Applied and Environmental Microbiology* **63**, 3352-3358.

Kotula, K.L. and Thelappurate, R. (1994) Microbiological and sensory attributes of retail cuts of beef treated with acetic and lactic acid solutions. *Journal of Food Protection* **57**, 665-670.

Krysinski, E.P., Brown, L.J. & Marchisello, T.J. (1992) Effect of cleaners and sanitisers on attached *L. monocytogenes* to product contact surfaces. *Journal of Food Protection* **55**, 246-251.

Lambert, A.D., Smith, J.P. and Dodds, K.L. (1991) Shelf life extension and microbiological safety of fresh meat - a review. *Food Microbiology* **8**, 267-297.

Lambert, J.D. and Maxcy, R.B. (1984) Effect of Gamma radiation on *Campylobacter jejuni*. *Journal of Food Science* **49**, 665-674.

Lawrence, J.R., Korber, D.R., Hoyle, B.D., Costerton, J.W. and Caldwell, D.E. (1991) Optical sectioning of microbial biofilms. *Journal of Bacteriology* **173**, 6558-6567.

LeChavellier, M.W., Cawthon, C.D. and Lee, R.G. (1988) Inactivation of biofilm bacteria. *Applied and Environmental Microbiology* **54**, 2492-2499.

Lee, R.M., Hartman, P.A., Olson, D.G. and Williams, F.D. (1994a) Metal ions reverse the inhibitory effects of selected food-grade phosphates in *Staphylococcus aureus*. *Journal of Food Protection* **57**, 284-288.

Lee, R.M., Hartman, P.A., Olson, D.G. and Williams, F.D. (1994b) Bactericidal and bacteriolytic effects of selected food-grade phosphates, using *Staphylococcus aureus* as a model system. *Journal of Food Protection* **57**, 276-283.

Lemcke, R.M. (1959) The changes with age in the resistance of *Escherichia coli* to drying under atmospheric conditions. *Journal of Applied Bacteriology* **22**, 253-257.

Lewin, R.(1984) Microbial adhesion is a sticky problem. *Science* **224**, 375-377.

Lillard, H.S (1980) Effect on broiler carcasses and water of treating chiller water with chlorine or chlorine dioxide. *Poultry Science* **59**, 1761-1766.

Lillard, H.S. (1985) Bacterial cell characteristics and conditions influencing their adhesion to poultry skin. *Journal of Food Protection* **48**, 803-807.

Lillard, H.S. (1986) Distribution of "attached" *Salmonella typhimurium* cells between poultry skin and a surface film following water immersion. *Journal of Food Protection* **49**, 449-454.

Lillard, H.S. (1988) Effect of surfactant or changes in ionic strength on the attachment of *Salmonella typhimurium* to poultry skin and muscle. *Journal of Food Science* **53**, 727-730.

Lillard, H.S. (1994) Effect of trisodium phosphate on salmonellae attached to food. *Journal of Food Protection* **57**, 465-469.

Liuzzo, J.S., Barone, W.B. and Novak, A.F. (1966) Stability of B-vitamins in Gulf oysters preserved by Gamma radiation. *Federal Proceedings*, **25**, 722.

Loaharanu, P. (1989) International trade in irradiated foods: Regional status and outlook. *Food Technology*, **43**, 77-80.

Mafu, A.A., Roy, D., Goulet, J. and Magny, P. (1990) Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene and rubber surfaces after short contact times. *Journal of Food Protection* **53**, 742-746.

Marshall, K.C. and Criuckshank, R.H. (1973) Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. *Archives of Microbiology* **91**, 29-40.

Marshall, K.C.(1985) Mechanisms of bacterial adhesion at solid-water interfaces. In Bacterial adhesion: mechanisms and physiological significance, ed. Savage, D.C. and Fletcher, M. pp. 133-161. New York: Plenum Press New York and London.

Marshall, K.C., Stout, R. and Mitchell, R.(1971) Mechanisms of initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology* **68**, 337-348.

Marshall, N.J., Goodwin, C.J. and Holt, S.J. (1995) A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. *Growth Regulation* **5**, 69-84.

Martin, M.L., Shipman, L.D., Wells, J.G., Potter, M.E., Hedberg, K., Wachsmuth, I.K., Tauxe, R.V., Davis, J.P., Arnolai, J. and Tilleli, J. (1986) Isolation of *Escherichia coli* O157:H7 from dairy cattle associated with two cases of hemolytic uremic syndrome. *Lancet* ii, 1043.

May, K.N. (1961) Skin contamination of broilers during commercial evisceration. *Poultry Science* **40**, 531-536.

McEldowney, S. and Fletcher, M (1986) Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. *Journal of General Microbiology* **132**, 513-523.

McEldowney, S. and Fletcher, M (1988) The effect of temperature and relative humidity on the survival of bacteria attached to dry solid surfaces. *Letters in Applied Microbiology* **7**, 83-86.

Meadows, P.S. (1971) The attachment of bacteria to solid surfaces. *Archives of Microbiology* 75, 374-381.

Mendonca, A.F., Amoroso, T.L. and Knabel, S.J. 1994. Destruction of Gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. *Applied and Environmental Microbiology* **60**, 4009-4014.

Meynell, G.G. and Meynell, E. (1970) Theory and Practice of Experimental Bacteriology (2nd Edition), pp.30-31. Cambridge: Cambridge University Press.

Miles, R. J., Cassar, C. and Carneiro de Melo, A. (1995) Bacterial decontamination of foods. Patent application: PCT/GB 96/03173. Publication date 3 July 1997.

Miller, A.J., Brown, T. and Call, J.E. (1996) Comparison of wood and polyethylene cutting boards: potential for the attachment and removal of bacteria from ground beef. *Journal of Food Protection* **59**, 854-858.

Monk, J.D., Beuchat, L.R. and Doyle, M.P. (1995) Irradiation inactivation of food-borne microrganisms. *Journal of Food Protection* **58**, 197-208.

Monk, J.D., Beuchat, L.R. and Hathcox, A.K. (1996). Inhibitory effects of sucrose monolaurate, alone and in combination with organic acids, on *Listeria monocytogenes* and *Staphylococcus aureus*. *Journal of Applied Bacteriology* **81**, 7-18.

Monticello, D.J. (1989) Control of microbial growth with nisin/lysozyme formulations. Eur. Pat. Appl. 89123445.2.

Morris, C.A., Lucia, L.M., Savell, J.W. and Acuff, G.R. (1997) Trisodium phosphate treatment of pork carcasses. *Journal of Food Protection* **62**,402-403, 405.

Mosteller, T.M. and Bishop, J.R. (1993) Sanitizer efficacy against attached bacteria in a milk biofilm. *Journal of Food Protection* **56**, 34-41.

Mullerat, J., Sheldon, B.W. and Klapes, N.A. (1995) Inactivation of *Salmonella* species and other food-borne pathogens with Salmide®, a sodium chlorite-based oxyhalogen disinfectant. *Journal of Food Protection* **58**, 535-540.

Musial, C.E. and Rosenblatt, J.E. (1989) Antimicrobial susceptibilities of anaerobic bacteria isolated at the Mayo Clinic during 1982 through 1987: comparison with results from 1977 through 1981. *Mayo Clinic Proceedings* **64**, 392-399.

Mustapha, A. and Leiwen, M.B. (1989) Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. *Journal of Food Protection* **52**, 306-311.

Nelson, J.H. (1990) Where are *Listeria* likely to be found in dairy plants? *Dairy, Food and Environmental Sanitation* **10**, 344-345.

Nikiado, H. and Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* **49**, 1-32.

Niskanen, A. and Pohja, M.S. (1977). Comparative studies on the sampling and investigation of microbial contamination of surfaces by the contact plate and swab methods. *Journal of Applied Bacteriology* **42**, 53-63.

Noller, E.C. and Hartsell, S.E. (1960) Bacteriolysis of *Enterobacteriaceae*. 1. Lysis by four lytic systems utilising lysozyme. *Journal of Bacteriology* **81**, 482-491.

Norde, W.(1986) Adsorption of proteins from solution at the solid-liquid interface. *Advances in Colloid and Interface Science* **25**, 267-340.

Notermans, S. and Kampelmacher, E.H. (1974) Attachment of some bacterial strains to the skin of broiler chickens. *British Poultry Science* **15**, 573-575.

Notermans, S., Dufrenne, J. and Van Schothorst, M. (1980) The effect of cultural procedures on the attachment of bacteria to chicken breast meat. *Journal of Applied Bacteriology* **49**, 273-279.

Oh, D. and Marshall, D.L. (1995) Monolaurin and acetic acid inactivation of *Listeria monocytogenes* attached to stainless steel. *Journal of Food Protection* **59**, 249-252.

Owen Fields, F. (1996) Use of bacteriocins in food: regulatory considerations. *Journal of Food Protection Supplement*, 72-77.

Palumbo, S.A. and Williams, A.C. (1990) Effect of temperature, relative humidity and suspending menstrua on the resistance of *Listeria monocytogenes* to drying. *Journal of Food Protection* **53**, 377-381.

Palumbo, S.A., Call, J.E., Cooke, P.H. and Williams, A.C. (1995). Effect of polyphosphates and NaCl on *Aeromonas hydrophila* K144. *Journal of Food Safety* **15**, 77-87.

Park, P.K. and Cliver, D.O. (1996) Disinfection of household cutting boards with a microwave oven. *Journal of Food Protection* **59**, 1049-1054.

Parry, P.T., Haysom, L. and Davies, R. (1982) A manual of recommended methods for microbiological examination of poultry and poultry products. British Poultry Meat Association Ltd. London.

Patterson, J.T. (1971) Microbiological assessment of surfaces. *Journal of Food Technology* **6**, 63-72.

Paul, J.H. and Jeffrey, W.H. (1985) Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio proteolytica*. *Applied and Environmental Microbiology* **50**, 431-437.

Pearson, J., Southam, G.G. and Holley, R.A. (1987) Survival and transport of bacteria in egg washwater. *Applied and Environmental Microbiology* **53**, 2060-2065.

Personal communication, (1995) Survey of contamination of surfaces in catering kitchens. Department of Health Steering Group on Microbiological Safety of Food, London.

Phillips, C.A. (1995) Incidence, epidemiology and prevention of foodborne *Campylobacter* species. *Trends in Food Science and Technology* **6**, 83-97.

Piette, J.-P.G. and Idziak, E.S. (1991) Role of flagella in adhesion of *Pseudomonas fluorescens* to tendon slices. *Applied and Environmental Microbiology* **57**, 1635-1639.

Piette, J.-P.G. and Idziak, E.S. (1992) A model study of factors involved in adhesion of *Pseudomonas fluorescens* to meat. *Applied and Environmental Microbiology* **58**, 2783-2791.

Pirt, S. J. (1975) Principles of microbe and cell cultivation. London: Blackwell Scientific Publications.

Pontefract, R.D. (1991) Bacterial Adherence: Its consequences in food processing. *Canadian Institute of Science and Technology Journal* **24**, 113-117.

Postgate, J. R. (1969) Viable counts and viability. *Methods in Microbiology* **1**, 611-628.

Prescott, L.M., Harley, J.P. and Klein, D.A. (1993) Microbiology (2nd Edition). Iowa: Wm. C. Brown Publishers; Dubuque.

Pringle, J.H. and Fletcher, M. (1983) Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Applied and Environmental Microbiology* **45**, 811-817.

Raloff, J. (1993) Wood wins: plastic trashed for cutting meat. *Science News* **143**, 84-85.

Rathgeber, B.M. and Waldroup, A.L. (1995) Antibacterial activity of a sodium acid pyrophosphate product in chiller water against selected bacteria on broiler carcasses. *Journal of Food Protection* **58**, 530-534.

Razavi-Rohani, S.M. and Griffiths, M.W. (1994) The effect of mono and polyglycerol laurate on spoilage and pathogenic bacteria associated with foods. *Journal of Food Safety* **14**, 131-151.

Repaske, R. (1958) Lysis of Gram -ve organisms and the role of versene. *Biochemica et Biophysica Acta* **30**, 225-232.

Robach, M.C. and Sofos, J.N. (1982) Use of sorbates in meat products, fresh poultry and poultry products: A review. *Journal of Food Protection* **45**, 374-378.

Roberts, D. (1990) Sources of infection: Food. *The Lancet* **336**, 859-861.

Rosenberg, M., Gutnick, D and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters* **9**, 29-33.

Ryan, C.A., Tauxe, R.V., Hosek, G.W., Wells, J.G., Stoesz, P.A., McFadden, H.W. Jr, Smith, P.W., Wright, G.F. and Blake, P.A. (1986) *Escherichia coli* O157:H7 diarrhea in a nursing home. Clinical, epidemiological and pathological findings. *Journal of Infectious Disease* **154**, 631-638.

Saltermann, A.T., Biasutti, M.A., Senz, A. and Garcia N.A. (1995) Influence of the pH on the photodynamic effect in lysozyme - a comparative kinetic study with sensitized photooxidation of isolated amino acids. *Amino Acids*, **9**, 123-134.

Salton, M.R.J. (1957) The properties of lysozyme and its action on microorganisms. *Bacteriological Reviews* **21**, 82-99.

Sasahara, K. and Zottola, E.A. (1993) Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. *Journal of Food Protection* **56**, 1022-1028.

Schlegel, H.G. (1990) The prokaryotic cell. In General Microbiology (6th Edition), pg.47. Cambridge: Cambridge University Press.

Schüller, F., Benz, R. and Sahl, H.-G. (1989) The peptide antibiotic subtilin acts by formation of voltage-dependent multi-state pores in bacterial and artificial membranes. *European Journal of Biochemistry* **182**, 181-186.

Schwach, T.S. and Zottola, E.A. (1982) Use of scanning electron microscopy to demonstrate microbial attachment to beef and beef contact surfaces. *Journal of Food Science* **47**, 1401-1405.

Selgas, D., Marín, M.L., Pin, C. and Casas, C. (1993) Attachment of bacteria to meat surfaces: A Review. *Meat Science* **34**, 265-273.

Sims, G.R., Glenister, D.A., Brocklehurst, T.F. and Lund, B.M. (1989) Survival and growth of food poisoning bacteria following inoculation into cottage cheese varieties. *International Journal of Food Microbiology* **9**, 173-195.

Skaliy, P. and Eagon, R.G. (1972) Effect of physiological age and state on survival of desiccated *Pseudomonas aeruginosa*. *Applied Microbiology* **24**, 763-767.

Skelley, G.C., Fandino, G.E., Haigler, J.H. and Sherard, R.C.Jr. (1985) Bacteriology and weight loss of pork carcasses treated with a sodium hypochlorite solution. *Journal of Food Protection* **48**, 578-581.

Slavik, M.F., Griffis, C.L., Li, Y. and Engler, P.V. (1991) Effect of electrical stimulation on bacterial contamination of chicken eggs. *Journal of Food Protection* **54**, 508-513.

Slavik, M.F., Kim, J., Pharr, M.D., Raben, D.P., Tsai, S. and Lobsinger, C.M. 1994. Effect of trisodium phosphate on *Campylobacter* attached to post-chill chicken carcasses. *Journal of Food Protection* **57**, 324-326.

Sleesman, J.P. and Leben, C. (1976) Bacterial desiccation: effects of temperature, relative humidity and culture age on survival. *Phytopathology* **66**, 1334-1338.

Smulders, F.J.M., Barendsen, P., van Logtestijn, J.G., Mossel, D.A.A. and van der Marel, G.M. (1986) Review: Lactic acid: considerations in favour of its acceptance as a meat decontaminant. *Journal of Food Technology* **21**, 419-436.

Sockett, P.N., Cowden, J.M., Le Baigue, S., Ross, D., Adak, G.K. and Evans, H. (1993) Foodborne disease surveillance in England and Wales: 1989-1991. In *Communicable Disease Report Review* 3, pp. R159-173. London: Public Health Laboratory Service.

Somers, E.B., Schoeni, J.L. and Wong, A.C.L. (1994). Effects of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni, Escherichia coli* 0157:H7, *Listeria monocytogenes* and *Salmonella typhimurium. International Journal of Food Microbiology* **22**, 269-276.

Speers, J.G.S. and Gilmour, A. (1985) The influence of milk and milk components on the attachment of bacteria to farm dairy equipment surfaces. *Journal of Applied Bacteriology* **59**, 325-332.

Speers, J.G.S., Gilmour, A., Fraser, T.W. and McCall, R.D. (1984) Scanning electron microscopy of dairy equipment surfaces contaminated by two milk-borne micro-organisms. *Journal of Applied Bacteriology* **57**, 139-145.

Spencer, J.V. and Smith, L.E. (1962) The effect of chilling chicken fryers in a solution of poly-phosphates upon moisture uptake, microbial spoilage, tenderness, juiciness and flavour. *Poultry Science* **41**, 1685-1686.

Sprenger, R.A.(1989) Hygiene for Management (4th Edition), pp. 130-145. London: HighfieldPublications.

Spurlock, A.T. and Zottola, E.A. (1991) Growth and attachment of *Listeria monocytogenes* to cast iron. *Journal of Food Protection* **54**, 925-928.

Stevens, K.A., Klapes, N.A., Sheldon, B.W. and Klaenhammer, T.R. (1992) Effect of treatment conditions on nisin inactivation of Gram-negative bacteria. *Journal of Food Protection* **55**, 763-766.

Stevens, R.A. and Holah, J.T. (1993) The effect of spray-wash temperature on bacterial retention on abraded domestic sink surfaces. *Journal of Applied Bacteriology* **75**, 91-94.

Stone, L.S and Zottola, E.A. (1985) Scanning electron microscopy study of stainless steel finishes used in food processing equipment. *Food Technology* **39**, 112-114.

Strange, R.E. and Cox, C.S. (1976) Survival of dried and airborne bacteria. In The survival of vegetative microbes ed. Gray, T.R.G. and Postgate, J.R. Society for General Microbiology Symposium 26, pp 111-154. Cambridge: Cambridge University Press.

Stringer, M.F. (1994) *Campylobacter*: a European perspective. *Dairy, Food and Environmental Sanitation* **14**, 325-329.

Suárez, B., Perreirós, C.M. and Criado, M. (1992) Adherence of psychrotrophic bacteria to dairy equipment surfaces. *Journal of Dairy Research* **59**, 381-388.

Sveum, W.H., Moberg, L.J., Rude, R.A and Frank, J,F. (1992) Microbiological monitoring of the food processing environment. In:Compendium of methods for the microbiological examination of foods, ed. Vanderzant, C. and Splittstoesser, D.F. pp 51-74. Washington, DC: American Public Health Association.

Tamblyn, K.C., Conner, D.E. and Bilgili,S.F. (1997) Utilization of the skin attachment model to determine the antibacterial efficacy of potential carcass treatments. *Poultry Science* **76**, 1318-1323.

Tappero, J.W., Schuchat, A., Deaver, K.A., Mascola, L. and Wenger, J.D. (1995) Reduction in the incidence of human listeriosis in the United States. *Journal of the American Medical Association* **273**, 1118-1122.

Thayer, D.W. and Boyd, G. (1995a) Radiation sensitivity of *Listeria monocytogenes* on beef as affected by temperature. *Journal of Food Science* **60**, 237-240.

Thayer, D.W., Boyd, G. and Huhtanen, C.N. (1995b) Effects of ionising radiation and anaerobic refrigerated storage on indigenous microflora, *Salmonella*, and *Clostridium botulinum* types A and B in vacuum-packed, mechanically deboned chicken meat. *Journal of Food Protection*, **58**, 752-757.

Thom, S.M., Horobin, R.W., Seidler,E. and Barer, M.R. (1993) Factors affecting the selection and uses of tetrazolium salts as cytochemical indicators of microbial viability and activity. *Journal of Applied Bacteriology* **74**, 433-443.

Thomas, A., Chart, H., Cheasty, T., Smith, J.E., Frost, J.A. and Rowe, B. (1993) Vero cytotoxigenic-producing *Escherichia coli*, particularly serogroup O157, associated with human infection in the United Kingdom: 1989-1991. *Epidemiology and Infection* **110**, 591-600.

Thomas, C.J. and McMeekin, T.A. (1981a) Attachment of *Salmonella* spp. to chicken muscle surfaces. *Applied and Environmental Microbiology*, **42**, 130-134.

Thomas, C.J. and McMeekin, T.A. (1981b) Contamination of broiler carcass skin during commercial processing procedure: an electron micrograph study. *Applied and Environmental Microbiology*, **42**, 134-144.

Thomas, C.J. and McMeekin, T.A. (1982) Effect of water immersion on the microtopography of the skin of chicken carcasses. *Journal of the Science of Food and Agriculture* **33**, 549-554.

Thomas, C.J. and McMeekin, T.A.(1984) Effect of water uptake by poultry tissues on contamination by bacteria during immersion in bacterial suspensions. *Journal of Food Protection* **47**, 398-402.

Threlfall, E.J., Hampton, M.D., Schofield, S.L., Ward, L.R., Frost, J.A. and Rowe, B. (1996) Epidemiological application of differentiating multiresistant *Salmonella typhimurium* DT104 by plasmid profile. In *Communicable Disease Report Review* 6, pp. R155-159. London: Public Health Laboratory Service.

Todd, E. (1991) Epidemiology of foodborne illness: North America. In Foodborne Illness, A Lancet Review ed. Waites, W.M. and Arbuthnott, J.P. pp. 9-15. London: Edward Arnold.

U. S. Department of Agriculture. (1982). Meat and poultry products: phosphates and sodium hydroxide. Federal Register. **47**: 10779.

U. S. Department of Agriculture. (1994). Use of Trisodium Phosphate on raw, chilled poultry carcasses (Title 9, Part 381). Federal Register. **59**, 551-554.

Unilever Industrial Product Information Sheet (Quatdet SU321), 1996.

Urbain, W.M. (1978) Food Irradiation. *Advances in Food Research*, **24**, 155-227.

Van de Weyer, A., Devleeschouwer, M.J. and Dony, J. (1993) Bactericidal activity of disinfectants on *Listeria*. *Journal of Applied Microbiology* **74**, 480-483.

van Loosedrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.B.J. (1989) Bacterial adhesion: a physico-chemical approach. *Microbial Ecology* **17**, 1-15.

van Loosdrecht, M.C.M., Lyklema, J., Norde, W. and Zehnder, A.B.J. (1990) Influence of interfaces on microbial activity. *Microbiological Reviews* **54**, 75-87.

van Netten, P., Mossel, D.A.A. and Huis In't Veld, J. (1995) Lactic acid decontamination of fresh pork carcasses: a pilot plant study. *International Journal of Food Microbiology* **25**, 1-9.

Verheyen. C.C.P.M., Dhert, W.J.A., de Blieck-Hogervorst, J.M.A., van der Reijden, T.J.K., Petit, P.L.C. and de Groot, K. (1993) Adherence to a metal, polymer and composite by *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Biomaterials* **14**, 383-391.

Wall, P.G., de Louvois, J., Gilbert, R.J. and Rowe, B. (1996) Food poisoning: notifications, laboratory reports, and outbreaks - where do the statistics come from and what do they mean? *Communicable Diseases Report Review* 6, R93-R100.

Ward, J.B. and Berkeley, R.C.W. (1980) The Microbial Cell Surface. In: *Microbial Adhesion to Surfaces*, ed. Berkeley, R.C.W., Lynch, J.M., Melling, J., Rutter, P.R. and Vincent, B. pp. 47-66. Chichester: Ellis Horwood, England.

Warren, G.H., Gray, J. and Yurchenco, J.A. (1957) Effect of polymyxin on the lysis of *Neisseria catarrhalis*. *Journal of Bacteriology* **74**, 788-793.

Welker, C., Faiola, N., Davis, S., Maffatore, I. and Batt, C.A. (1997) Bacterial retention and cleanability of plastic and wood cutting boards with commercial food service maintenance practices. *Journal of Food Protection* **60**, 4078-413.

Wells, J.G., Davis, B.R., Wachsmuth, I.K., Riley, L.W., Remis, R.S., Sokow, R. and Morris, G.K. (1983) Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. *Journal of Clinical Microbiology* **18**, 512-520.

WHO (1992) Surveillance Programme for Control of Foodborne Infections and Intoxications in Europe. Fifth Report 1985-1989. Berlin: Robert von Ostertag Institute, FAO/WHO Collaborating Centre.

Wick, E., Murray, E., Mizutani, J. and Koshika, M. (1967) Irradiation flavour and the volatile components of beef. In Radiation Preservation of Foods, pp 12-25. Washington, DC: American Chemical Society.

Wilson, M. (1996) Susceptibility of oral bacterial biofilms to antimicrobial agents. *Journal of Medical Microbiology* **44**, 79-87.

Winston, P.W. and Bates, D.H. (1960) Saturated solutions for the control of humidity in biological research. *Ecology* **41**, 232-237.

Wirtanen, G. and Mattila-Sandholm, T. (1992a) Removal of foodborne biofilms - Comparison of surface and suspension tests. Part I. *Lebensm. Wiss. u. Technol.* **25**, 43-49.

Wirtanen, G. and Mattila-Sandholm, T. (1992b) Effect of the growth phase of foodborne biofilms on their resistance to a chlorine sanitizer. Part II. *Lebensm. Wiss. u. Technol.* **25**, 50-54.

Wirtanen, G. and Mattila-Sandholm, T. (1993) Epifluorescence image analysis and cultivation of foodborne biofilm bacteria grown on stainless steel surfaces. *Journal of Food Protection* **56**, 678-683.

Witholt, B.H., Van Leerikhuisen, H. and De Leij, L. (1976) How does lysozyme penetrate through the bacterial outer membrane? *Biochemica et Biophysica Acta* **443**, 534-544.

Wolfson, L.M., Sumner, S.S. and Froning, G.W. (1994) Inhibition of *Salmonella typhimurium* on poultry by the lactoperoxidase system. *Journal of Food Safety* **14**, 53-62.

Wrangstadh, M., Conway, P.L. and Kjelleberg, S. (1986) The production and release of extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. *Archives of Microbiology* **145**, 220-227.

Yang, T.S and Cunningham, F.E. (1993) Stability of egg white lysozyme in combination with other antimicrobial substances. *Journal of Food Protection* **56**, 153-156.

Yu, F.P. and McFeters, G.A. (1994) Physiological responses of bacteria in bioflims to disinfection. *Applied and Environmental Microbiology*, **60**, 2462-2466.

Zeitoun, A.A.M., Debevere, J.M. and Mossel, D.A.A. (1994) Significance of *Enterobacteriaceae* as index organisms for hygiene on fresh untreated poultry, poultry treated with lactic acid and poultry stored in a modified atmosphere. *Food Microbiology* **11**, 169-176.

Zepeda, C.M., Kastner, C.L., Willard, B.L., Phebus, R.K., Schwenke, J.R., Fijal, B.A. and Prasai, R.K. (1994) Gluconic acid as a fresh beef decontaminant. *Journal of Food Protection*, **57**, 956-962.

Zhuang, R.-Y. and Beuchat, L. R.. (1996) Effectiveness of trisodium phosphate for killing *Salmonella montevideo* on tomatoes. *Letters in Applied Microbiology*. **22**: 97-100.

Zhuang, R.-Y., Beuchat, L. R. and Angulo, F.A. (1995) Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine. *Applied and Environmental Microbiology*. **61**: 2127-2131.

ZoBell, C.E. (1943) The effect of solid surfaces upon bacterial activity. *Journal of Bacteriology* **46**, 39-56.

Zoltai, P.T., Zottola, E.A. and McKay, L.L (1981) Scanning electron microscopy of microbial attachment to milk contact surfaces. *Journal of Food Protection* **44**, 204-208.

Zoltai, P.T., Zottola, E.A. and McKay, L.L. (1981) Scanning electron microscopy of microbial attachment to milk and milk contact surfaces. *Journal of Food Protection*, **44**, 204-208.

Zottola, E.A. (1991) Characterisation of the attachment matrix of *Pseudomonas fragi* attached to non-porous surfaces. *Biofouling* **5**, 37-55.

Zottola, E.A. and Sasahara, K.C. (1994) Microbial biofilms in the food processing industry - should they be a concern? *International Journal of Food Microbiology* **23**, 125-148.